PCT

FORM PTO-1390 (REV 11-98)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 227-135
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) Unknown 09/622976
INTERNATIONAL APPLICATION NO. PCT/FI99/00152	INTERNATIONAL FILING DATE 26 February 1999	PRIORITY DATE CLAIMED 27 February 1998

TITLE OF INVENTION
SELF-REPLICATING VECTOR FOR DNA IMMUNIZATION AGAINST HIV

APPLICANT(S) FOR DO/EO/US
TÄHTINEN et al

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has **NOT** expired.
   d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. To 16. Below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ Other items or information.

09622976 .100600

534 Rec'd PCT/PTO 25 AUG 2000

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/622976 Unknown	PCT/FI99/00152	227-135

17. ☒ The following fees are submitted:

	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5):	
-- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $970.00	
-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $840.00	
-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2) paid to USPTO $690.00	
-- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) $670.00	
-- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $96.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 840.00
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).	$ 130.00

CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE		
Total Claims	15	-20 =	0	X	$18.00	$ 0.00
Independent Claims	1	-3 =	0	X	$78.00	0.00
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)				$260.00		$ 0.00

TOTAL OF ABOVE CALCULATIONS =	$	**970.00**
Reduction by ½ for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 C.F.R. 1.9, 1.27, 1.28).		0.00
SUBTOTAL =	$	**970.00**
Processing fee of $130.00, for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)). +		0.00
TOTAL NATIONAL FEE =	$	**970.00**
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). **$40.00** per property +	$	0.00
Fee for Petition to Revive Unintentionally Abandoned Application ($1210.00 – Small Entity = $605.00)	$	0.00
TOTAL FEES ENCLOSED =	$	**970.00**
	Amount to be: refunded	$
	Charged	$

a. ☒ A check in the amount of $970.00 to cover the above fees is enclosed.
b. ☐ Please charge my Deposit Account No. 14-1140 in the amount of $_____ to cover the above fees. A duplicate copy of this form is enclosed.
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.
d. ☒ The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application.

NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

NIXON & VANDERHYE P.C.
1100 North Glebe Road, 8th Floor
Arlington, Virginia 22201
Telephone: (703) 816-4000

SIGNATURE

Arthur R. Crawford
NAME

25,327 REGISTRATION NUMBER

August 25, 2000 Date

09/622976

534 Rec'd PCT/PTO 25 AUG 2000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

#1/2 Amdt. A

In re Patent Application of

TÄHTINEN et al 09/622,976

Serial No. ~~**Unknown**~~

National Phase of **PCT/FI99/00152**

Filed: **August 25, 2000**

For: **SELF-REPLICATING VECTOR FOR DNA IMMUNIZATION AGAINST HIV**

Atty. Ref.: **227-135**

Group: 1648

Examiner: A. Salimi

* * * * * * * * * * *

August 25, 2000

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

PRELIMINARY AMENDMENT

Prior to calculation of the filing fee and in order to place the above identified application in better condition for examination, please amend the claims as follows:

IN THE CLAIMS

Claim 3, line 1, delete "or 2".

Claim 4, line 1, delete "any of the preceding claims" and insert -- claim 1 --.

Claim 6, line 2, delete "any of claims 1-5" and insert -- claim 1 --.

Claim 8, lines 1 and 2, delete "any of claims 1-5" and insert -- claim 1 --.

Claim 10, line 1, delete "any of claims 1-5" and insert -- claim 1 --.

Claim 12, line 3, delete "any of claims 1-5" and insert -- claim 1 --.

Claim 14, lines 1 and 2, delete "any of claims 1-5" and insert -- claim 1 --.

TÄHTINEN et al
Serial No. **Unknown**

REMARKS

The above amendments are made to place the claims in a more traditional format.

Respectfully submitted,
NIXON & VANDERHYE P.C.
By: _____
Arthur R. Crawford
Reg. No. **25,327**

ARC:lmy

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A Transfection of CHO415 cells with BPV-1 origin containing plasmids

↓

1–4 day — extraction of episomal DNA (short term replication assay)

↓

5–21 day — transfected cells are selected with G418

↓

Colonies are pooled or picked, expanded to cell lines under non-selective conditions for 2 weeks

↓

Episomal or total DNA is extracted and replication competence of plasmids is estimated (long term replication assay)

B

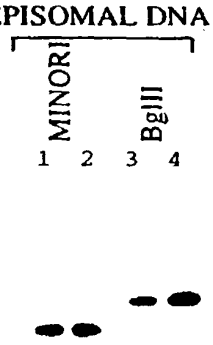


Fig. 1

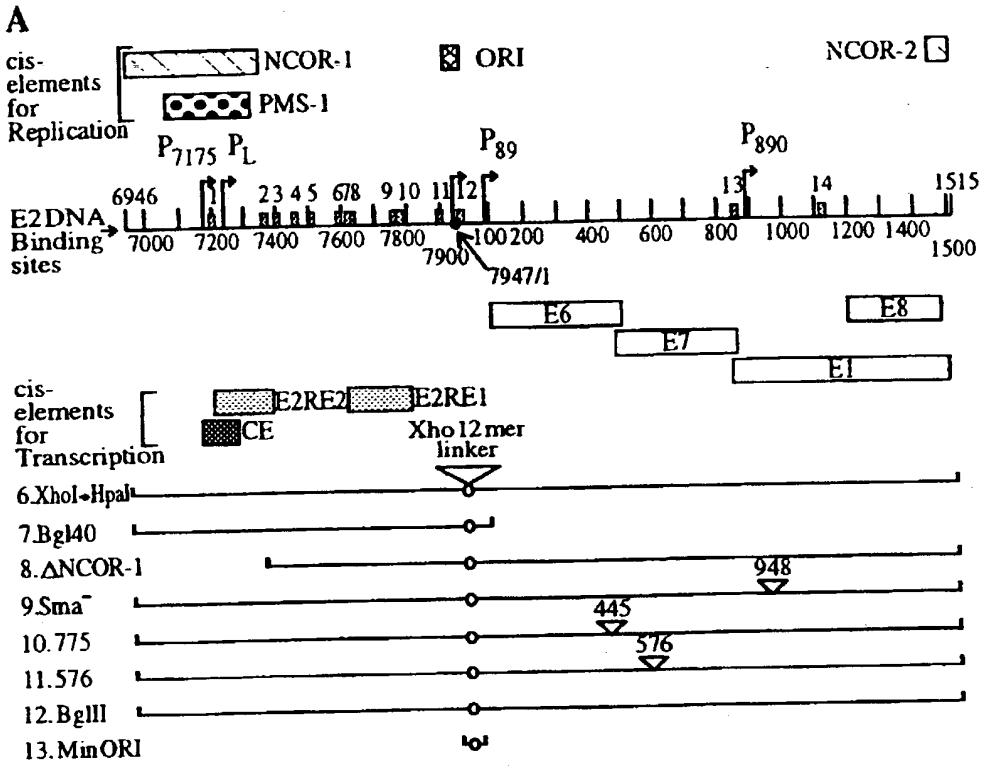

A
cis-elements for Replication
NCOR-1
ORI
NCOR-2
PMS-1
P7175 PL
P89
P890
6946
1 2 3 4 5 6 7 8 9 10 11 12 13 14
1515
E2DNA Binding sites
7000 7200 7400 7600 7800 7900 100 200 400 600 800 1000 1200 1400 1500
7947/1
E6
E8
E7
E1
cis-elements for Transcription
E2RE2
E2RE1
CE
Xho12mer linker
6.XhoI•HpaI
7.Bgl40
8.ΔNCOR-1
9.Sma⁻
10.775
11.576
12.BglII
13.MinORI
948
445
576
B
MARKER
EPISOMAL DNA
1 vector
2 Bgl40
3 BglII
4 —
5 vector
6 XhoI→HpaI
7 Bgl40
8 ΔNCOR
9 Sma⁻
10 775
11 576
12 BglII
13 MIN ORI

Fig. 2A, B

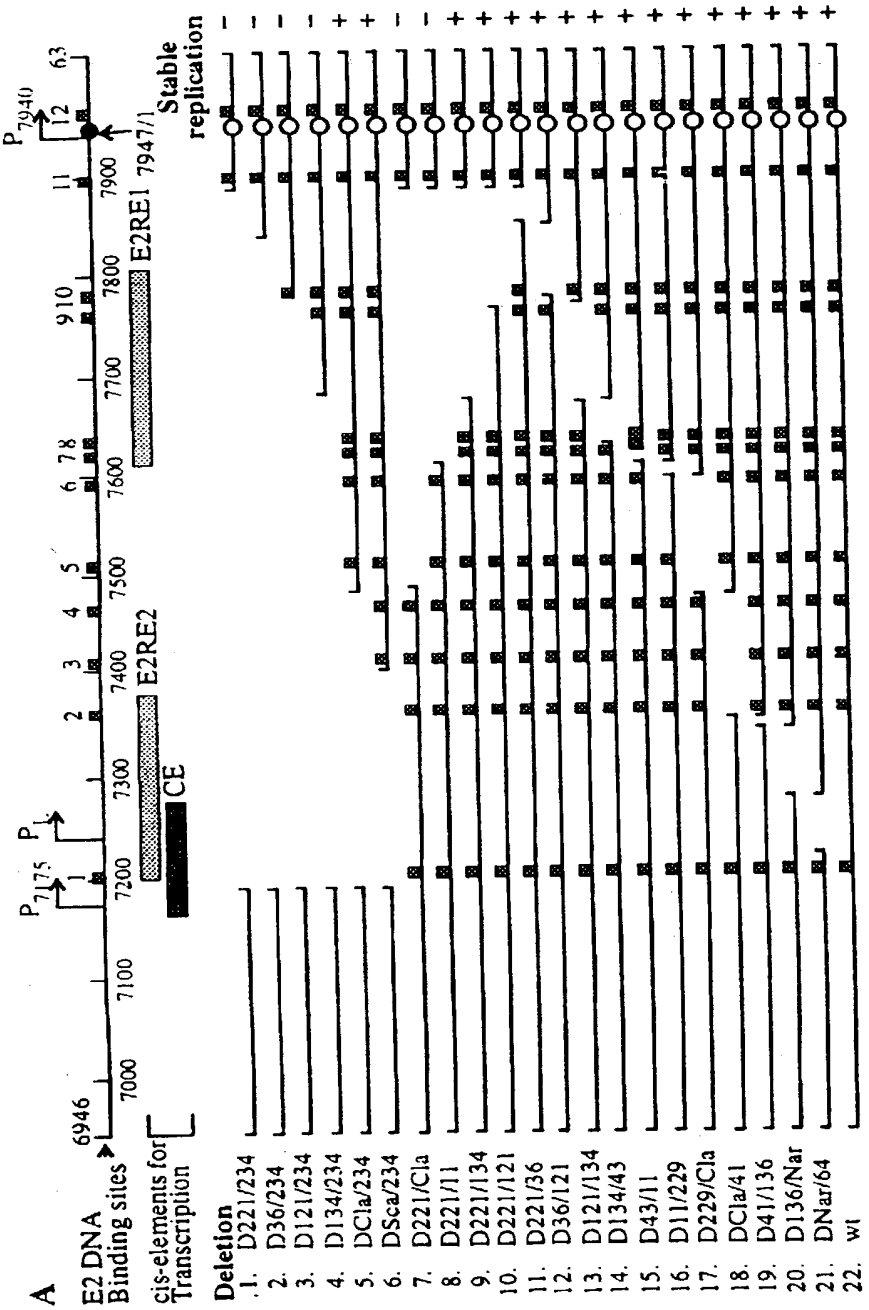

A
E2 DNA Binding sites
cis-elements for Transcription
CE
E2RE2
E2RE1
Stable replication
Deletion
1. D221/234
2. D36/234
3. D121/234
4. D134/234
5. DCla/234
6. DSca/234
7. D221/Cla
8. D221/11
9. D221/134
10. D221/121
11. D221/36
12. D36/121
13. D121/134
14. D134/43
15. D43/11
16. D11/229
17. D229/Cla
18. DCla/41
19. D41/136
20. D136/Nar
21. DNar/64
22. wt

Fig. 3A

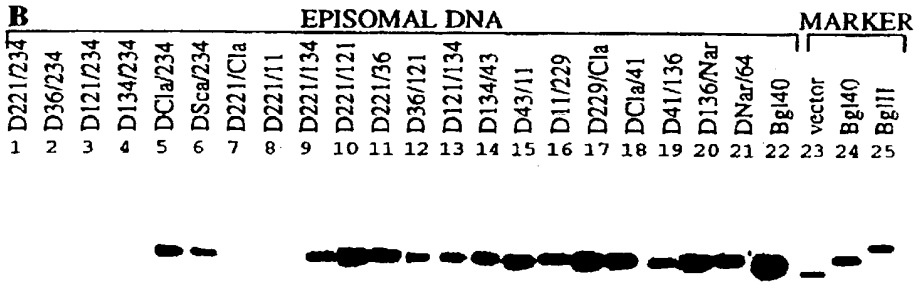
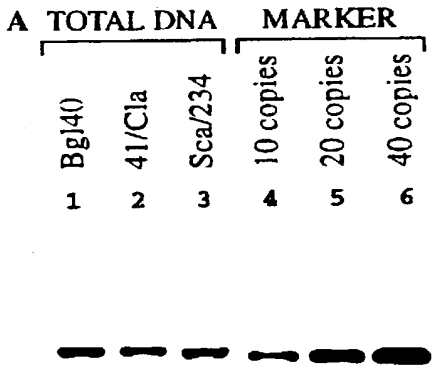


Fig. 3B



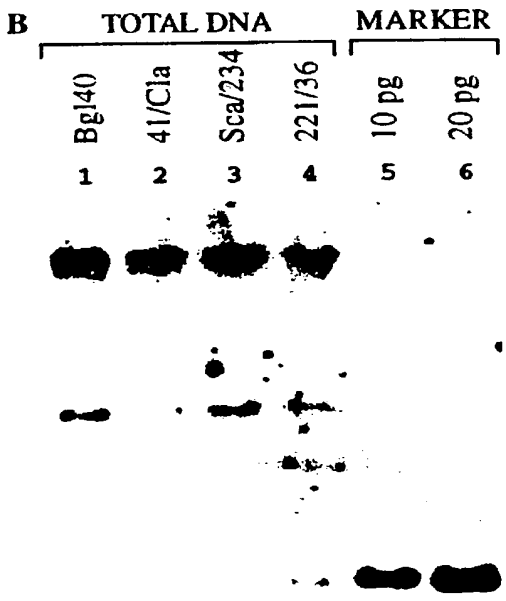


Fig. 4A, B

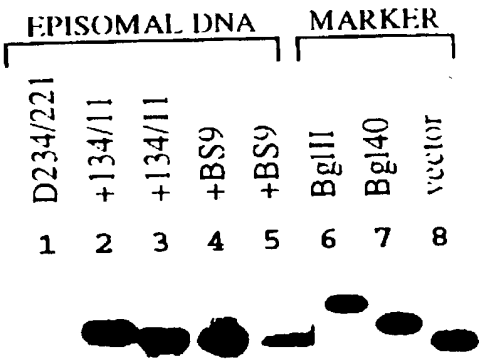

EPISOMAL DNA
MARKER
D234/221
+134/11
+134/11
+BS9
+BS9
BglII
Bgl40
vector
1 2 3 4 5 6 7 8

Fig. 5

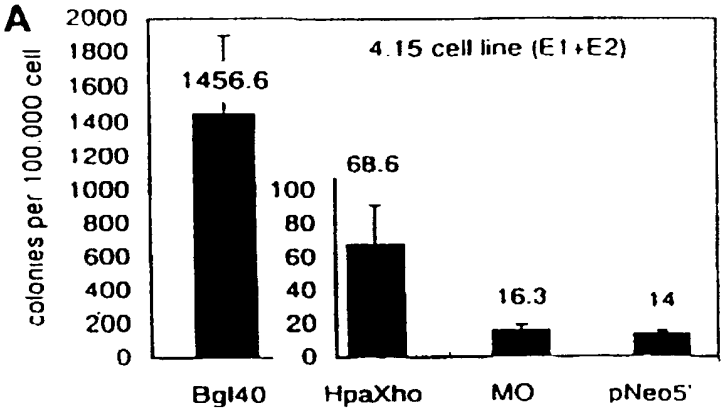

A
colonies per 100.000 cell
2000
1800
1600
1400
1200
1000
800
600
400
200
0
4.15 cell line (E1+E2)
1456.6
68.6
16.3
14
100
80
60
40
20
0
Bgl40
HpaXho
MO
pNeo5'

Fig. 6A

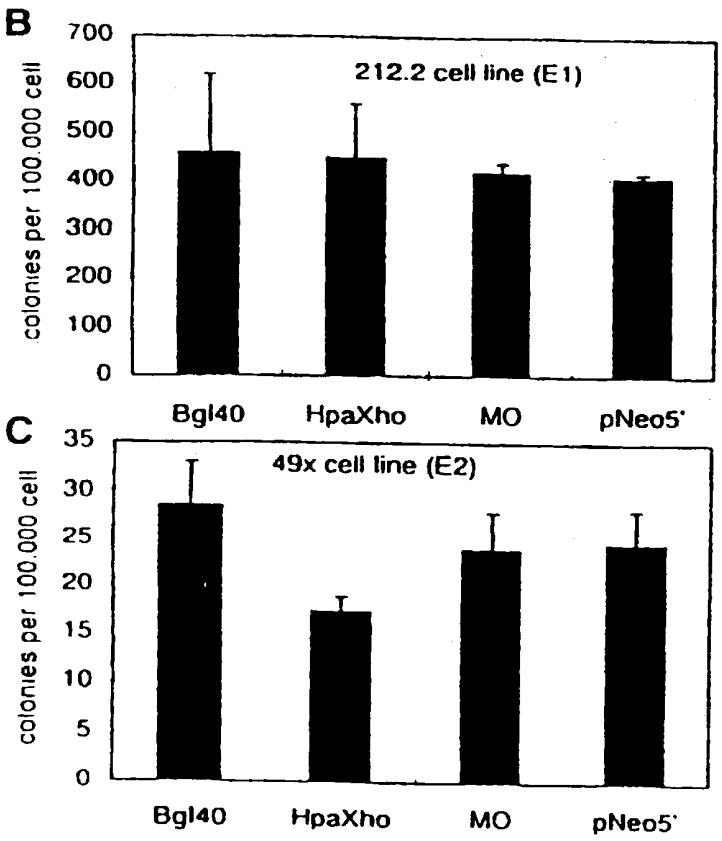

B
700
600
500
400
300
200
100
0
colonies per 100.000 cell
212.2 cell line (E1)
Bgl40
HpaXho
MO
pNeo5'
C
35
30
25
20
15
10
5
0
colonies per 100.000 cell
49x cell line (E2)
Bgl40
HpaXho
MO
pNeo5'

Fig. 6B, C

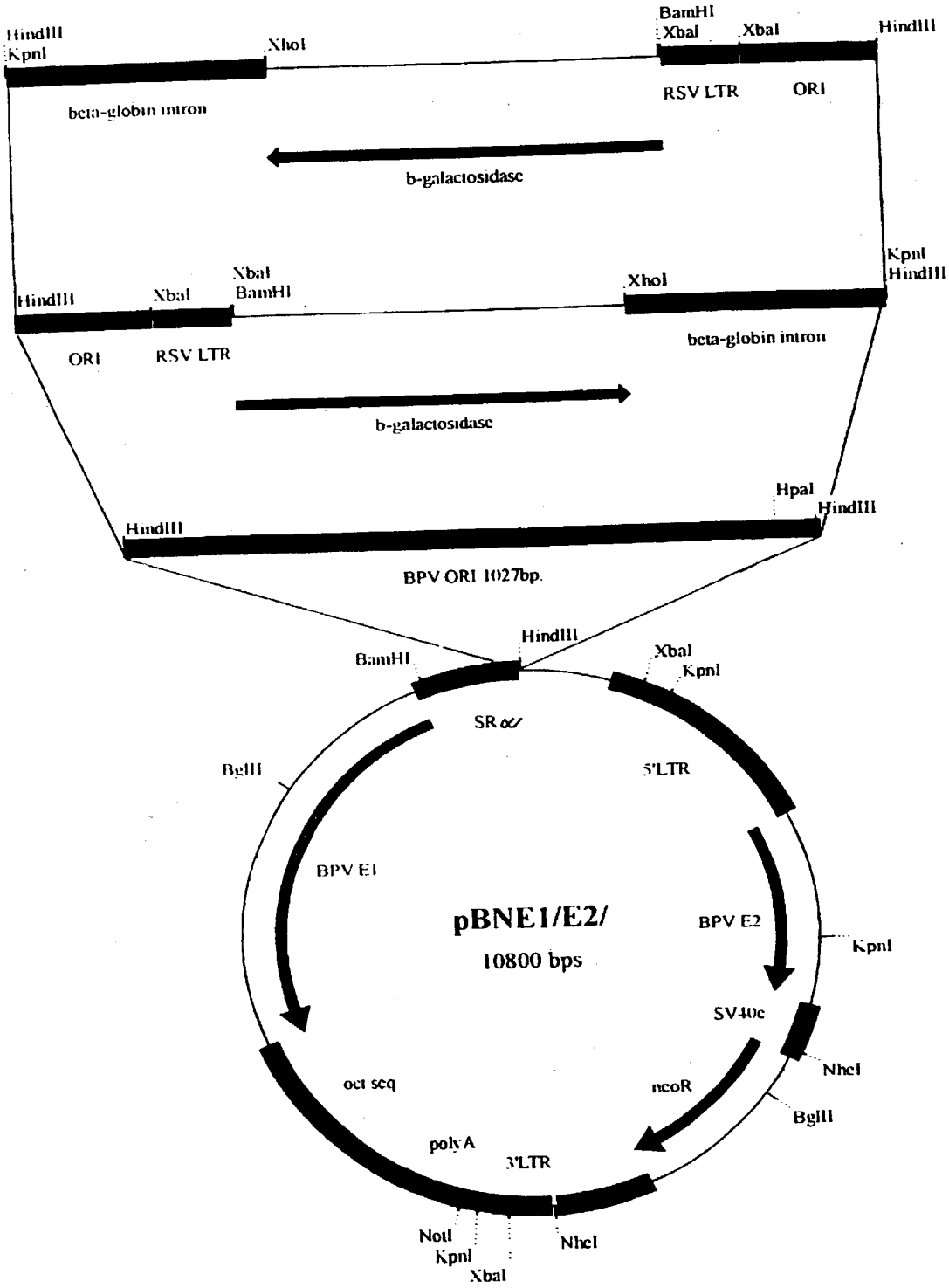

HindIII
KpnI
XhoI
BamHI
XbaI
XbaI
HindIII
beta-globin intron
RSV LTR
ORI
b-galactosidase
KpnI
HindIII
XbaI
BamHI
XhoI
HindIII
XbaI
beta-globin intron
ORI
RSV LTR
b-galactosidase
HpaI
HindIII
HindIII
BPV ORI 1027bp.
HindIII
BamHI
XbaI
KpnI
SR α
BglII
5'LTR
BPV E1
pBNE1/E2/
10800 bps
BPV E2
KpnI
SV40e
NheI
BglII
oct seq
ncoR
polyA
3'LTR
NotI
KpnI
XbaI
NheI

Fig. 7A

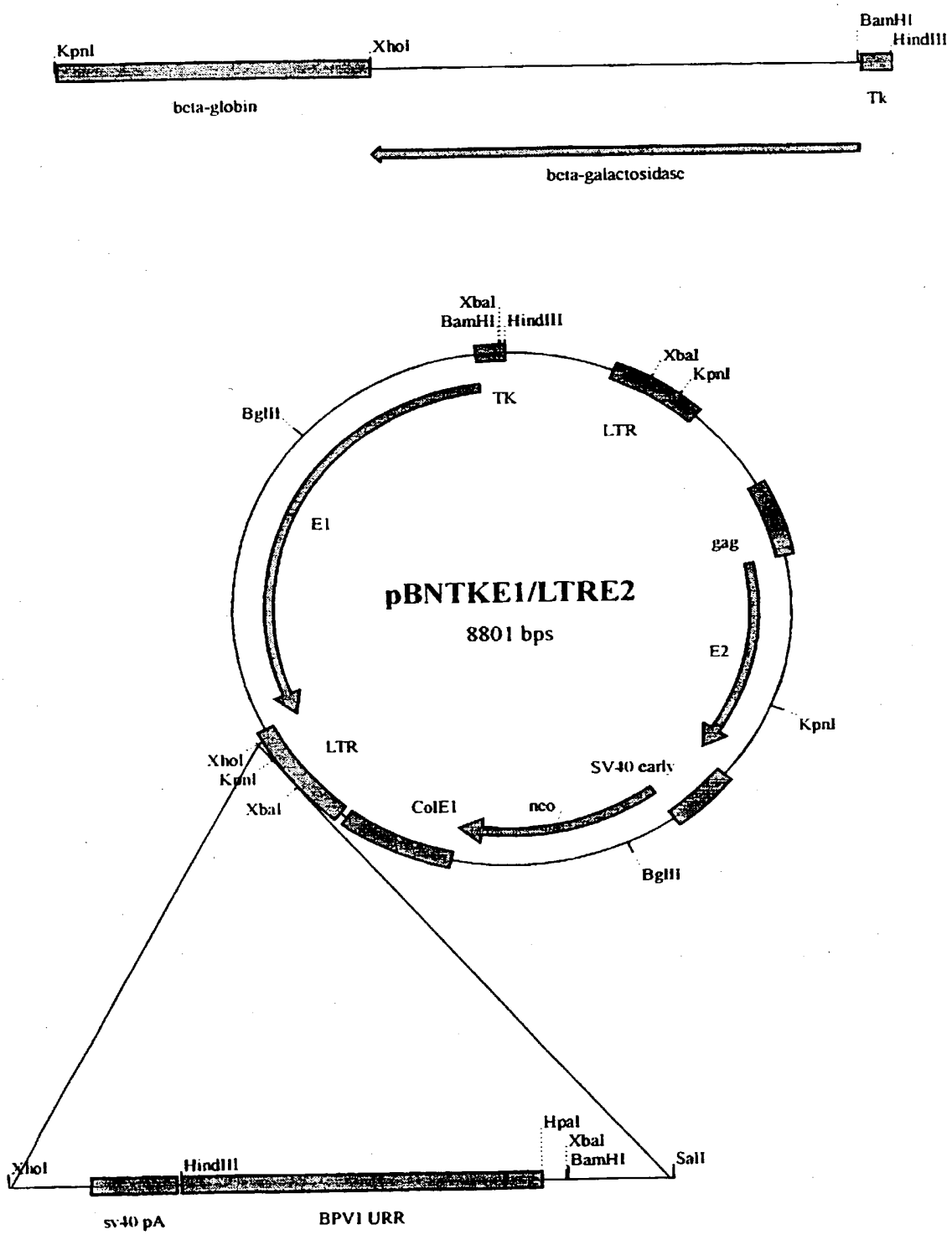

KpnI
XhoI
BamHI
HindIII
beta-globin
Tk
beta-galactosidase
XbaI
BamHI
HindIII
XbaI
KpnI
TK
LTR
BglII
E1
gag
pBNTKE1/LTRE2
8801 bps
E2
KpnI
LTR
XhoI
KpnI
XbaI
SV40 early
ColE1
neo
BglII
HpaI
XbaI
BamHI
SalI
XhoI
HindIII
sv40 pA
BPV1 URR

Fig. 7B

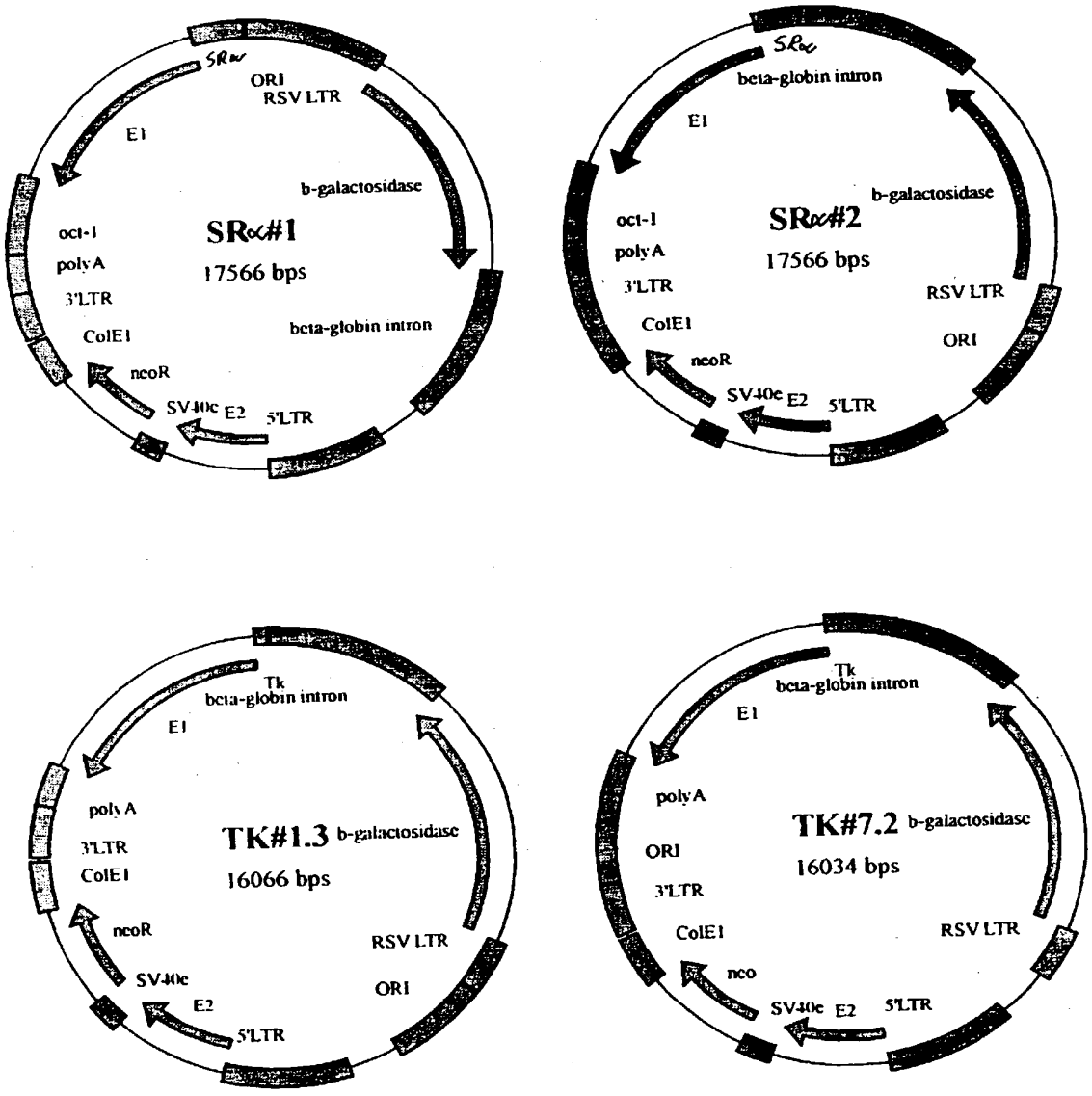
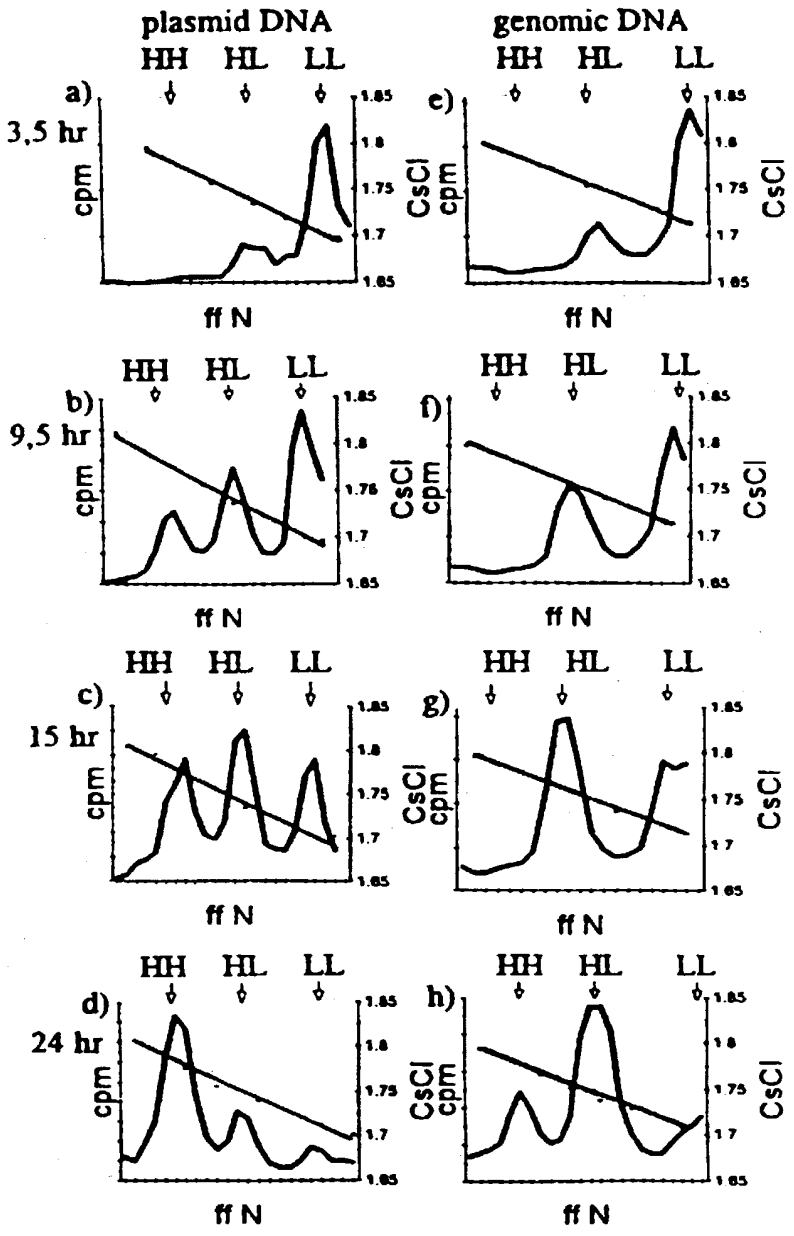

SRα#1
17566 bps
ORI
RSV LTR
E1
b-galactosidase
oct-1
poly A
3'LTR
ColE1
beta-globin intron
neoR
SV40e
E2
5'LTR
SRα#2
17566 bps
beta-globin intron
E1
b-galactosidase
oct-1
poly A
3'LTR
ColE1
RSV LTR
ORI
neoR
SV40e
E2
5'LTR
TK#1.3
16066 bps
Tk
beta-globin intron
E1
poly A
3'LTR
ColE1
b-galactosidase
neoR
SV40e
E2
5'LTR
RSV LTR
ORI
TK#7.2
16034 bps
Tk
beta-globin intron
E1
poly A
ORI
3'LTR
ColE1
b-galactosidase
neo
SV40e
E2
5'LTR
RSV LTR

Fig. 7C



	plasmid DNA			genomic DNA		
	HH	HL	LL	HH	HL	LL
3,5 hr	5%	19%	76%		27%	73%
9,5 hr	21%	34%	45%		44%	56%
15 hr	34%	38%	28%		58%	42%
24 hr	66%	24%	10%	24%	59%	17%

Fig. 8

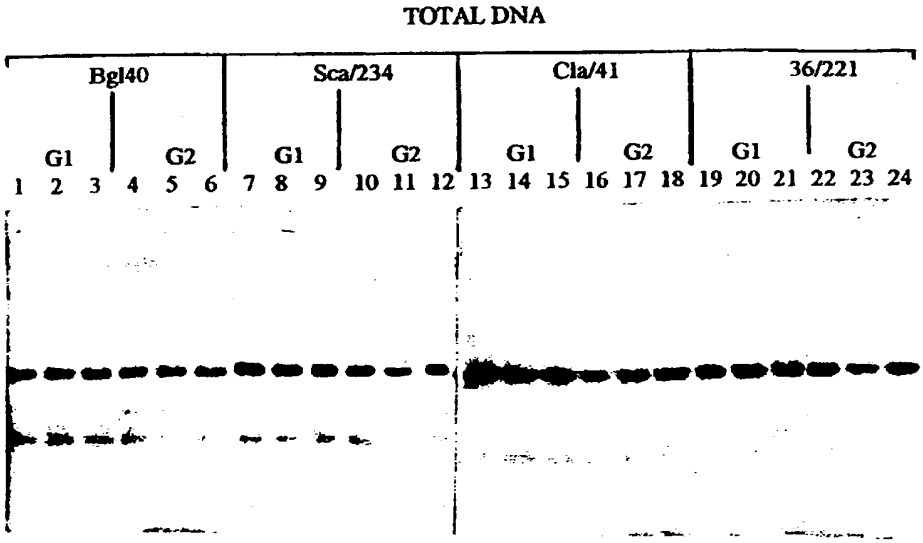

TOTAL DNA
Bgl40
Sca/234
Cla/41
36/221
G1
G2
G1
G2
G1
G2
G1
G2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Fig. 9

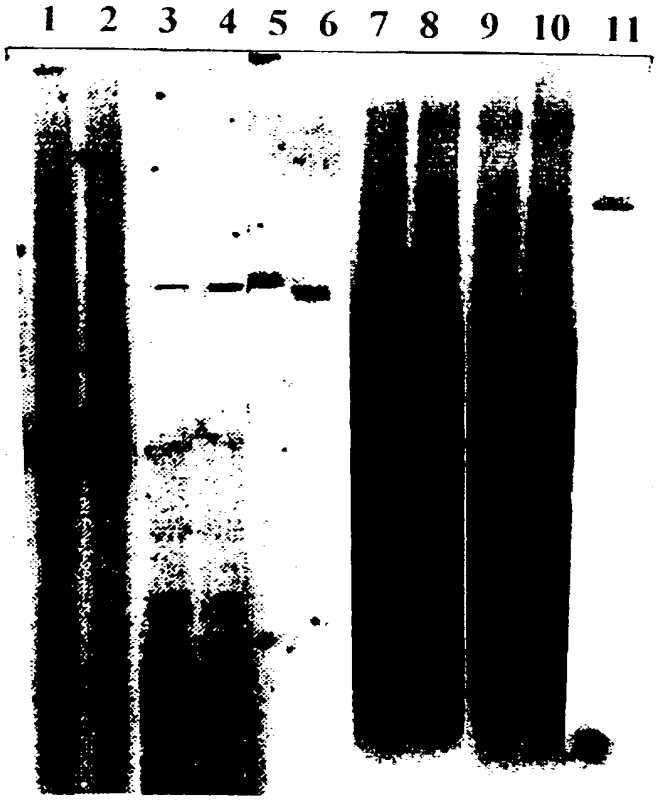


Fig. 10

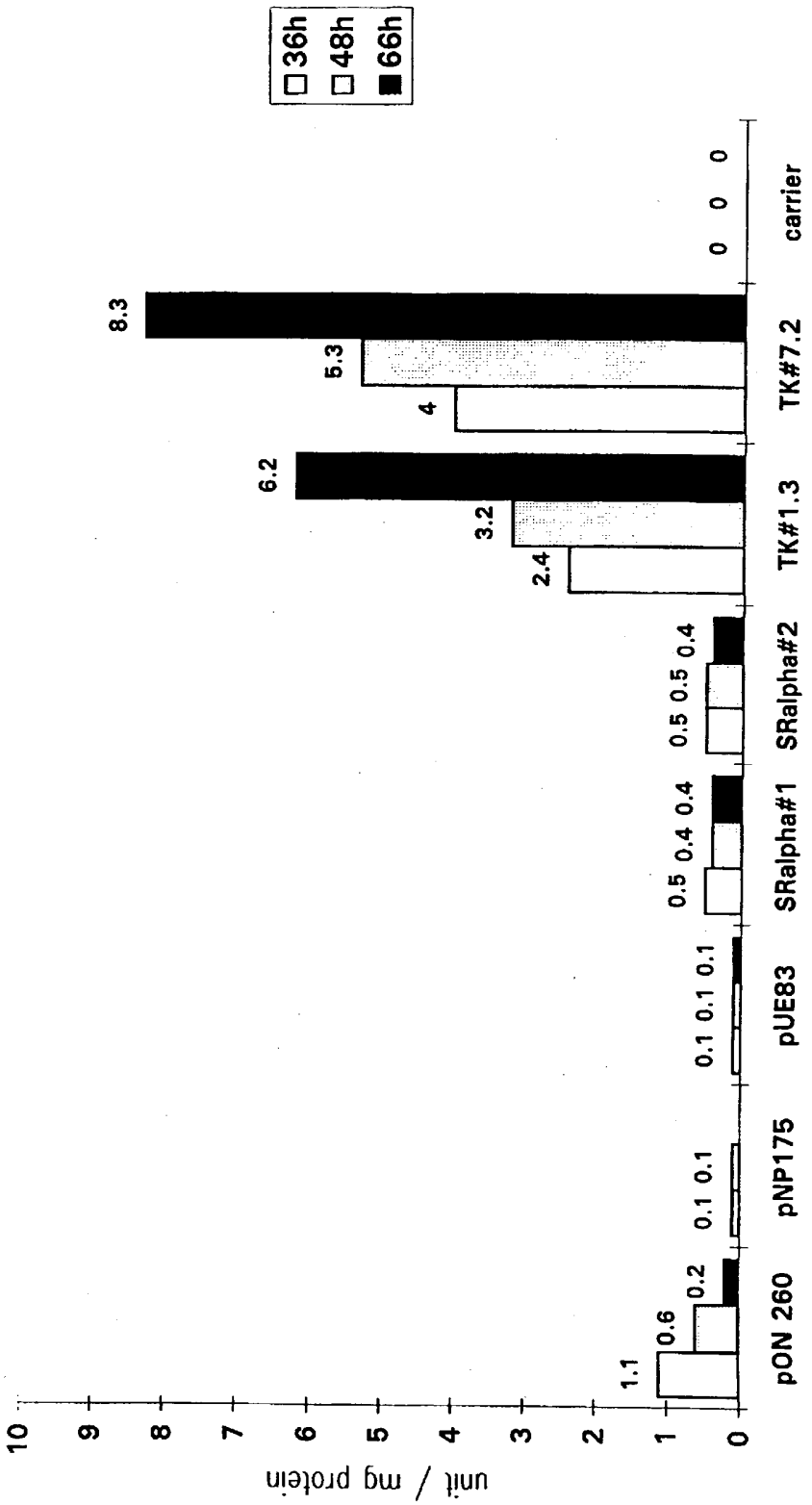

36h
48h
66h
unit / mg protein
0
1
2
3
4
5
6
7
8
9
10
1.1
0.6
0.2
0.1 0.1
0.1 0.1 0.1
0.5 0.4 0.4
0.5 0.5 0.4
2.4
3.2
6.2
4
5.3
8.3
0 0 0
pON 260
pNP175
pUE83
SRalpha#1
SRalpha#2
TK#1.3
TK#7.2
carrier

Fig. 11

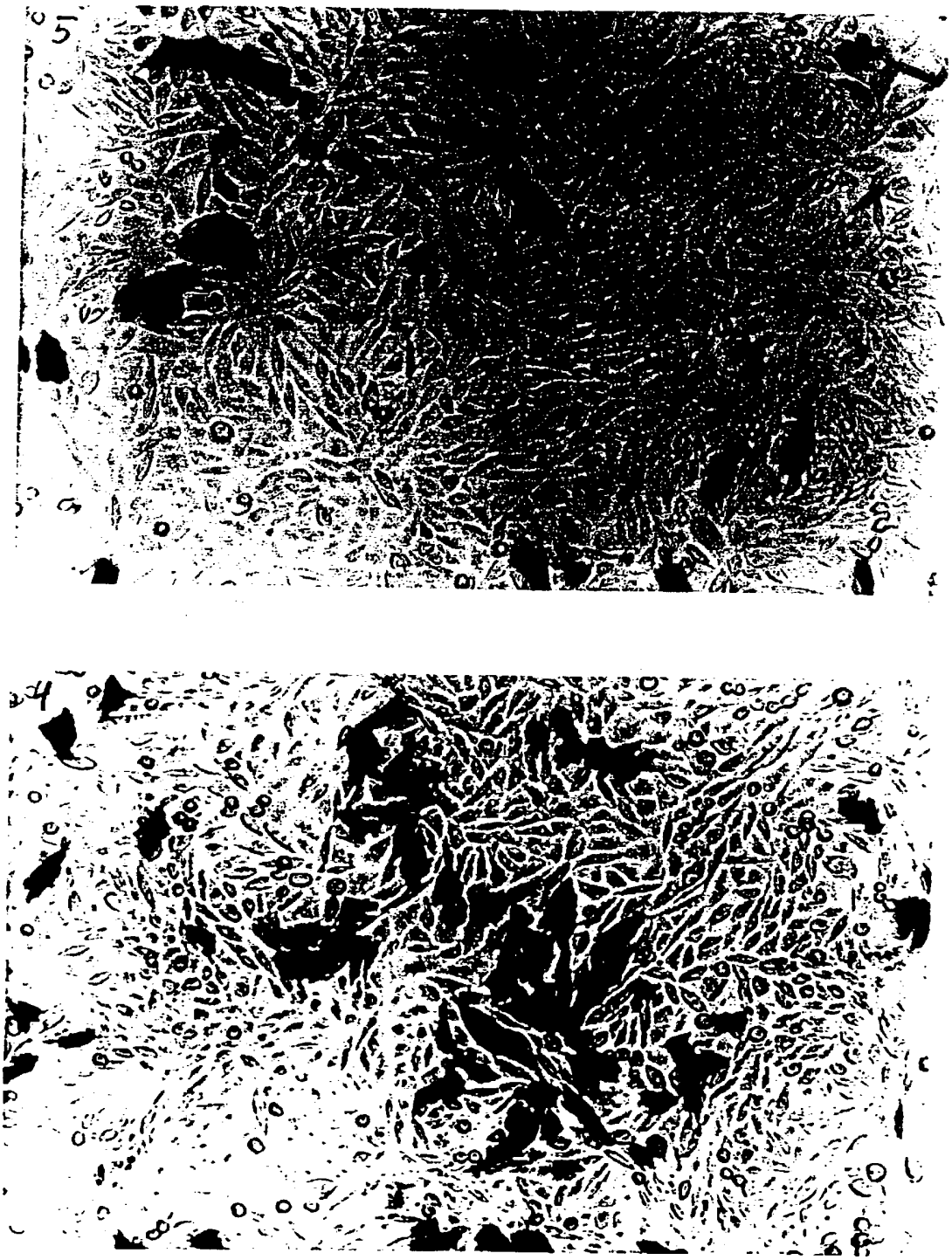


Fig. 12

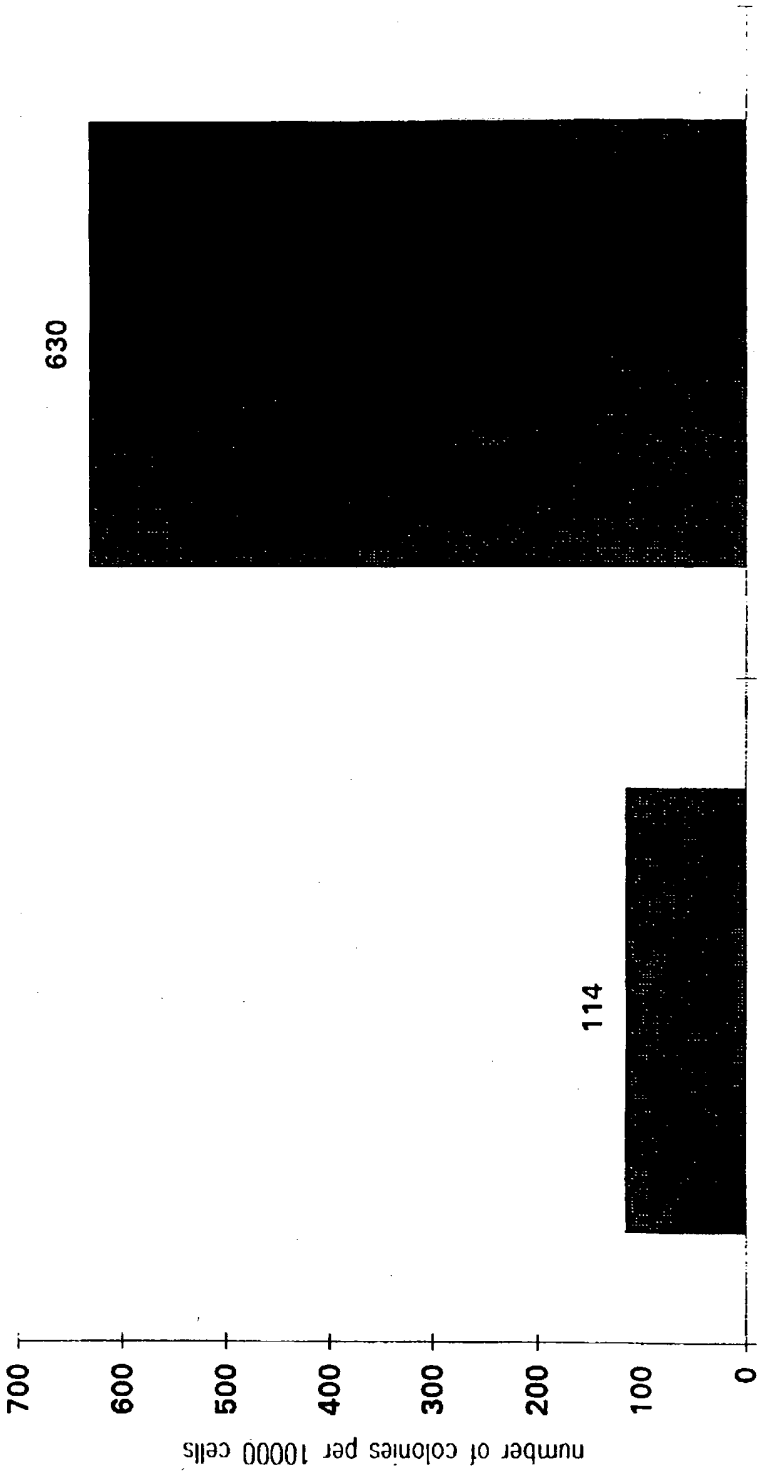

630
TK #1.3
114
SRalpha #1
700
600
500
400
300
200
100
0
number of colonies per 10000 cells

Fig. 13A

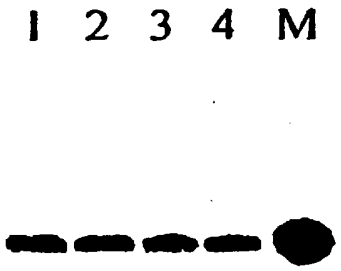


Fig. 13B

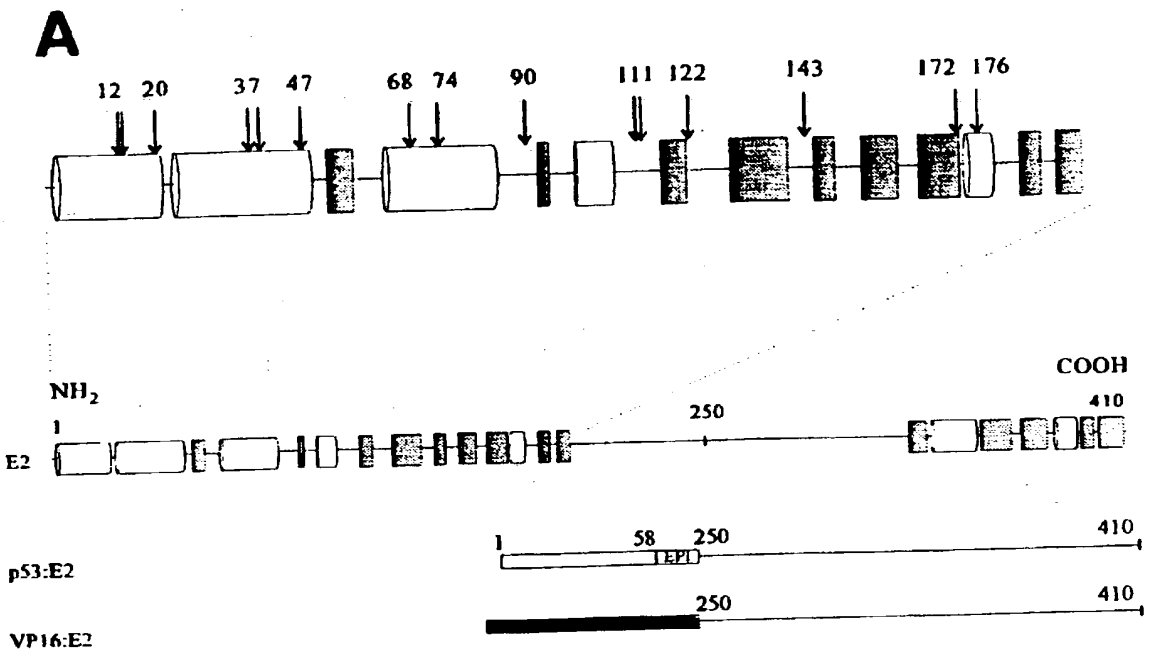


Fig. 14A

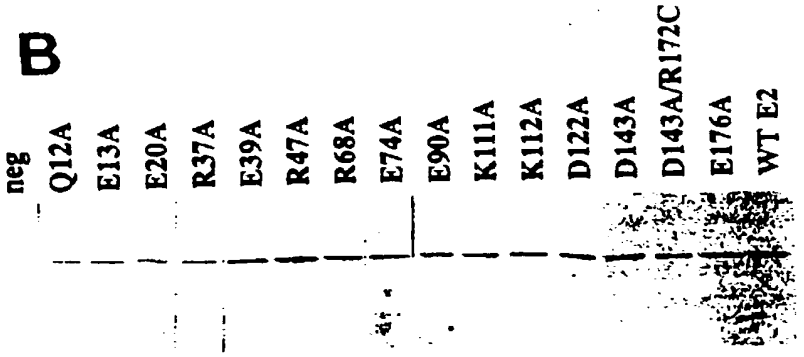


Fig. 14B

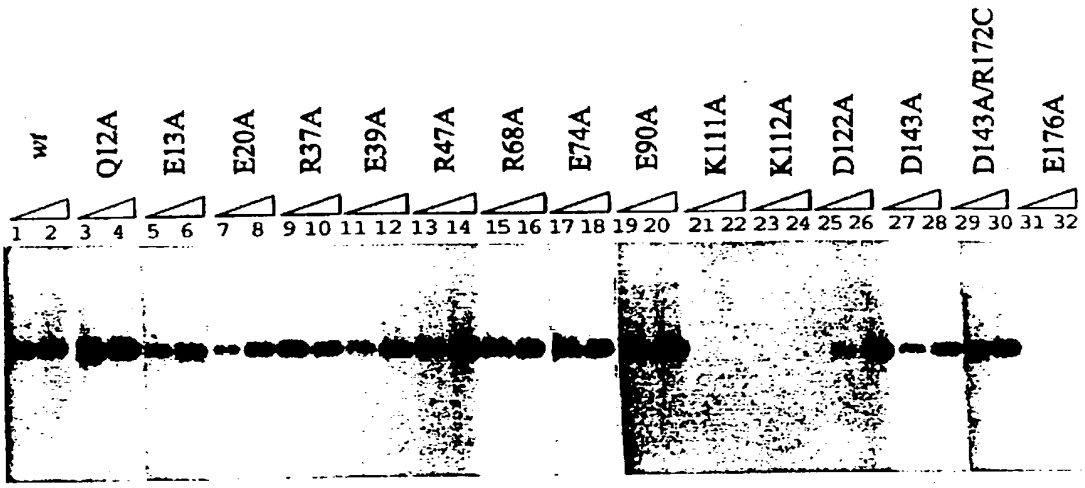


Fig. 15A

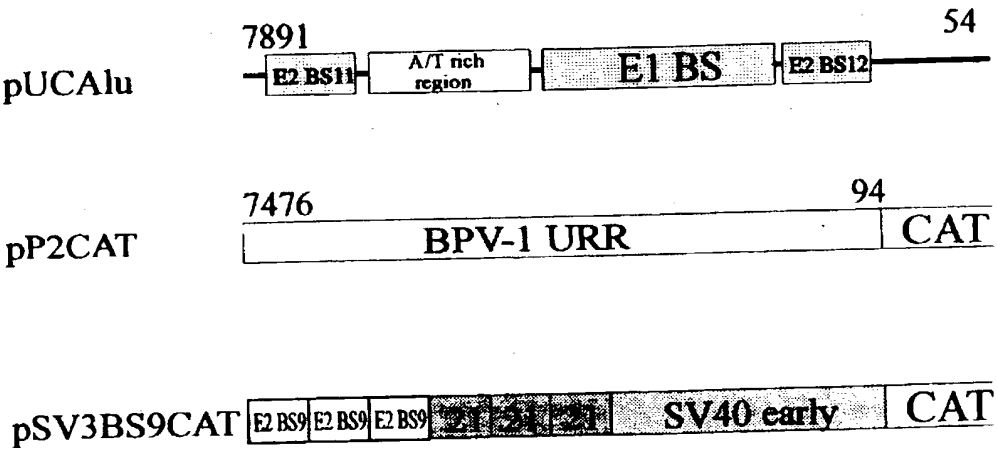


Fig. 15B

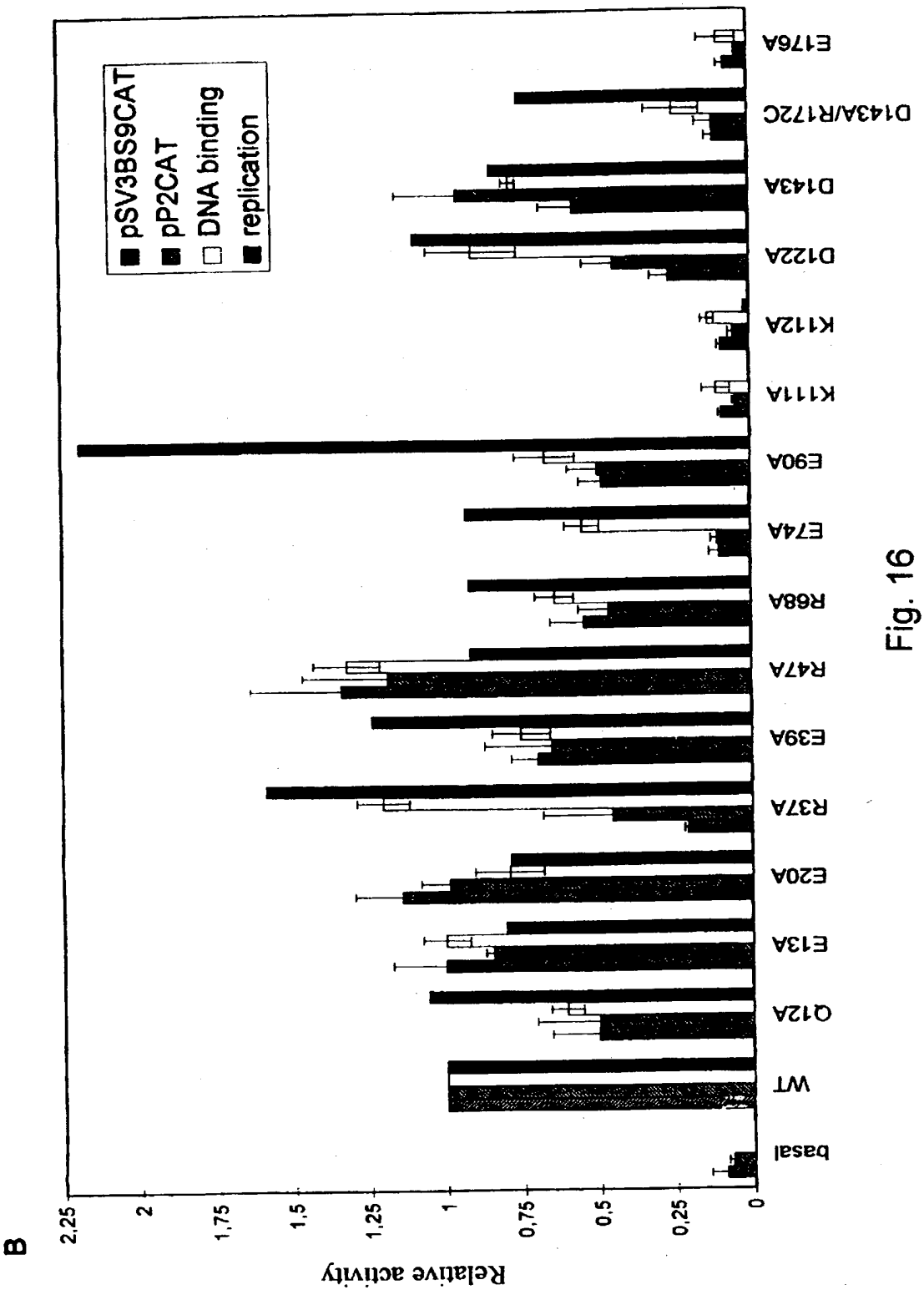

B
pSV3BS9CAT
pP2CAT
DNA binding
replication
Relative activity
2,25
2
1,75
1,5
1,25
1
0,75
0,5
0,25
0
basal
WT
Q12A
E13A
E20A
R37A
E39A
R47A
R68A
E74A
E90A
K111A
K112A
D122A
D143A
D143A/R172C
E176A

Fig. 16

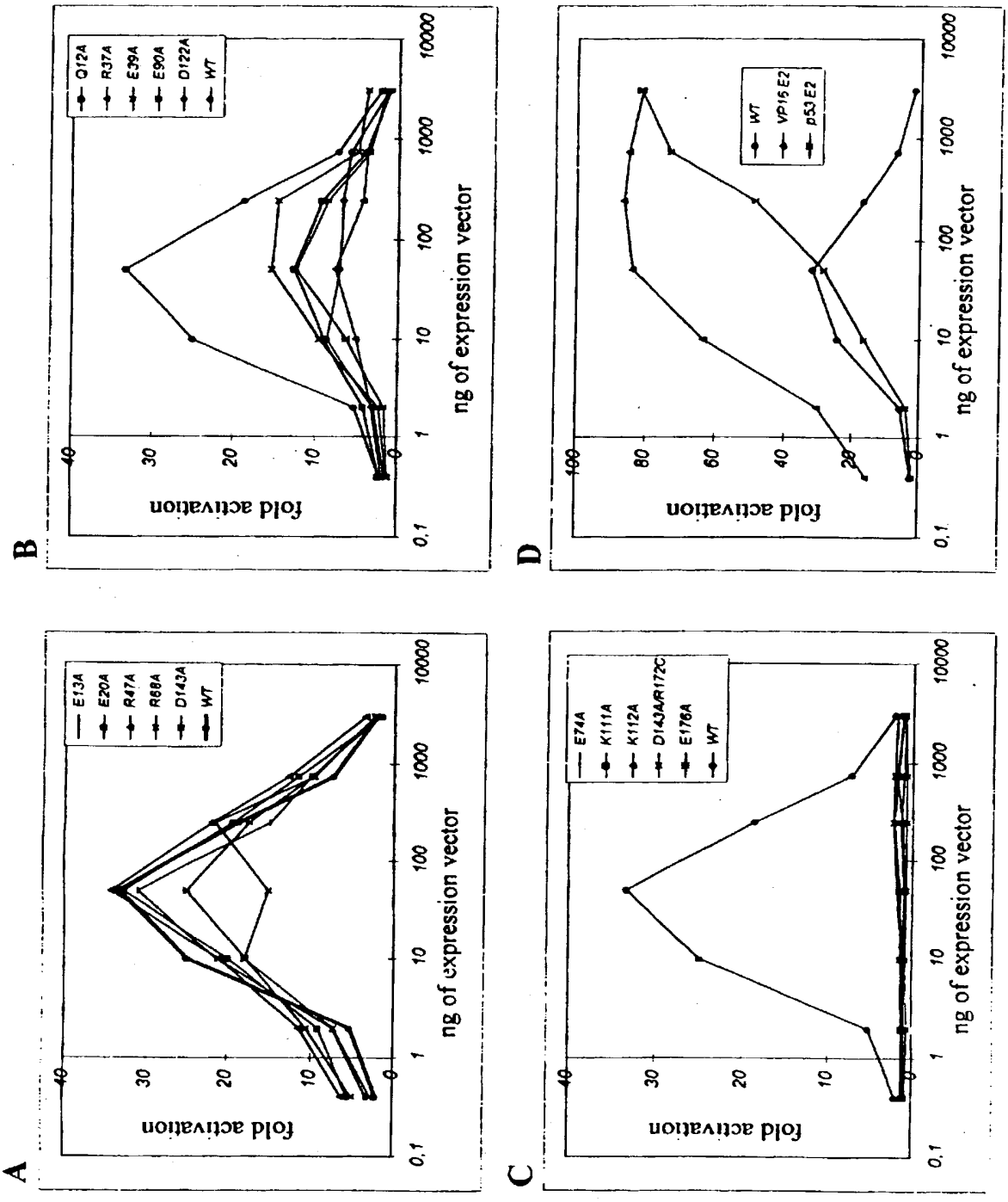

A
B
C
D
fold activation
ng of expression vector
0,1
1
10
100
1000
10000
E13A
E20A
R47A
R68A
D143A
WT
Q12A
R37A
E39A
E90A
D122A
E74A
K111A
K112A
D143A/R172C
E176A
VP16 E2
p53 E2

Fig. 17A, B, C, D

Fig. 18A, B

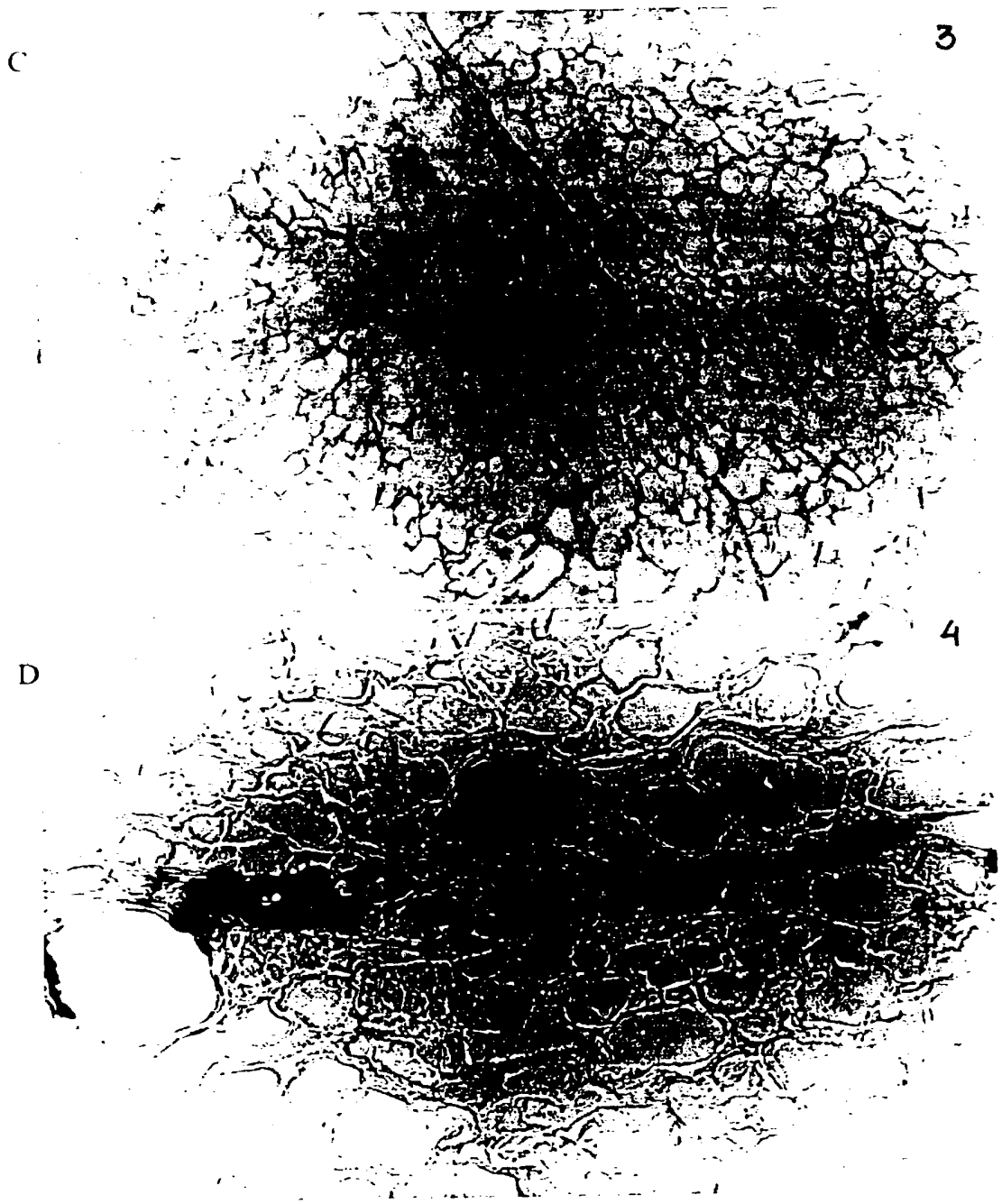


Fig. 18C, D

Fig. 18E, F

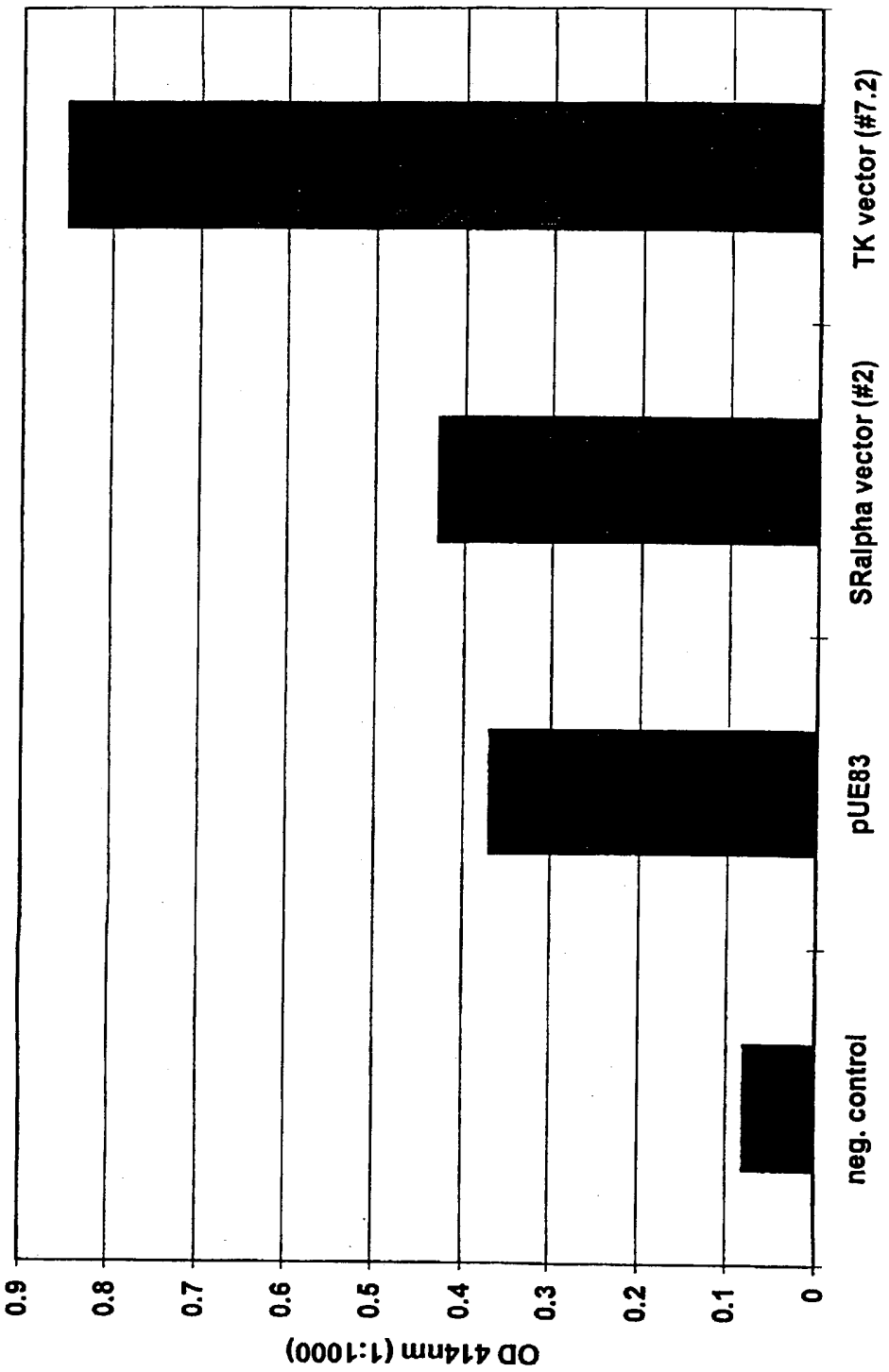

OD 414nm (1:1000)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
neg. control
pUE83
SRalpha vector (#2)
TK vector (#7.2)

Fig. 19

```
2600          2610          2620          2630          2640
  |             |             |             |             |
GTG AAG AGG ATG GAG ACA GCA TGC GAA CGT TTA CAT GTA GCG CAA
Val Lys Arg MET Glu Thr Ala Cys Glu Arg Leu His Val Ala Gln
                          1

      2650          2660          2670          2680
        |             |             |             |
GAA ACA CAA ATG CAG TTG ATT GAG AAA AGT AGT GAT AAG TTG CAA
Glu Thr Gln MET Gln Leu Ile Glu Lys Ser Ser Asp Lys Leu Gln
13

2690          2700          2710          2720          2730
  |             |             |           **  |             |
GAT CAT ATA CTG TAC TGG ACT GCT GTT AGA ACT GAG AAC ACA CTG
Asp His Ile Leu Tyr Trp Thr Ala Val Arg Thr Glu Asn Thr Leu
28

      2740          2750          2760          2770
        |             |             |             |
CTT TAT GCT GCA AGG AAA AAA GGG GTG ACT GTC CTA GGA CAC TGC
Leu Tyr Ala Ala Arg Lys Lys Gly Val Thr Val Leu Gly His Cys
43

2780          2790          2800          2810          2820
  |             |             |             |             |
AGA GTA CCA CAC TCT GTA GTT TGT CAA GAG AGA GCC AAG CAG GCC
Arg Val Pro His Ser Val Val Cys Gln Glu Arg Ala Lys Gln Ala
58

      2830          2840          2850          2860
      *   |         |             |             |
ATT GAA ATG CAG TTG TCT TTG CAG GAG TTA AGC AAA ACT GAG TTT
Ile Glu MET Gln Leu Ser Leu Gln Glu Leu Ser Lys Thr Glu Phe
73

2870          2880          2890          2900          2910
  |             |             |             |             |
GGG GAT GAA CCA TGG TCT TTG CTT GAC ACA AGC TGG GAC CGA TAT
Gly Asp Glu Pro Trp Ser Leu Leu Asp Thr Ser Trp Asp Arg Tyr
88

      2920          2930          2940          2950
        |             |             |             |
ATG TCA GAA CCT AAA CGG TGC TTT AAG AAA GGC GCC AGG GTG GTA
MET Ser Glu Pro Lys Arg Cys Phe Lys Lys Gly Ala Arg Val Val
103
```

Fig. 20

```
2960          2970          2980          2990          3000
  |             |             |             |             |
GAG GTG GAG TTT GAT GGA AAT GCA AGC AAT ACA AAC TGG TAC ACT
Glu Val Glu Phe Asp Gly Asn Ala Ser Asn Thr Asn Trp Tyr Thr
118

     3010          3020          3030          3040
       |             |             |             |
GTC TAC AGC AAT TTG TAC ATG CGC ACA GAG GAC GGC TGG CAG CTT
Val Tyr Ser Asn Leu Tyr MET Arg Thr Glu Asp Gly Trp Gln Leu
133

3050          3060          3070          3080          3090
  |             |             |             |             |
GCG AAG GCT GGG GCT GAC GGA ACT GGG CTC TAC TAC TGC ACC ATG
Ala Lys Ala Gly Ala Asp Gly Thr Gly Leu Tyr Tyr Cys Thr MET
148

     3100          3110          3120          3130
       |             |             | *           |
GCC GGT GCT GGA CGC ATT TAC TAT TCT CGC TTT GGT GAC GAG GCA
Ala Gly Ala Gly Arg Ile Tyr Tyr Ser Arg Phe Gly Asp Glu Ala
163

3140          3150          3160          3170          3180
  |             |             |             |             |
GCC AGA TTT AGT ACA ACA GGG CAT TAC TCT GTA AGA GAT CAG GAC
Ala Arg Phe Ser Thr Thr Gly His Tyr Ser Val Arg Asp Gln Asp
178

     3190          3200          3210          3220
       |             |             |             |
AGA GTG TAT GCT GGT GTC TCA TCC ACC TCT TCT GAT TTT AGA GAT
Arg Val Tyr Ala Gly Val Ser Ser Thr Ser Ser Asp Phe Arg Asp
193


3230          3240          3250          3260          3270
  |             |             |             |             |
CGC CCA GAC GGA GTC TGG GTC GCA TCC GAA GGA CCT GAA GGA GAC
Arg Pro Asp Gly Val Trp Val Ala Ser Glu Gly Pro Glu Gly Asp
208

     3280          3290          3300          3310
       |             |             |             |
CCT GCA GGA AAA GAA GCC GAG CCA GCC CAG CCT GTC TCT TCT TTG
Pro Ala Gly Lys Glu Ala Glu Pro Ala Gln Pro Val Ser Ser Leu
223
```

Fig. 20

```
3320          3330          3340          3350          3360
  |             |             |             |             |
CTC GGC TCC CCC GCC TGC GGT CCC ATC AGA GCA GGC CTC GGT TGG
Leu Gly Ser Pro Ala Cys Gly Pro Ile Arg Ala Gly Leu Gly Trp
238

      3370          3380          3390          3400
        |             |             |             |
GTA CGG GAC GGT CCT CGC TCG CAC CCC TAC AAT TTT CCT GCA GGC
Val Arg Asp Gly Pro Arg Ser His Pro Tyr Asn Phe Pro Ala Gly
253

3410          3420          3430          3440          3450
  |             |             |             |             |
TCG GGG GGC TCT ATT CTC CGC TCT TCC TCC ACC CCC GTG CAG GGC
Ser Gly Gly Ser Ile Leu Arg Ser Ser Ser Thr Pro Val Gln Gly
268

      3460          3470          3480          3490
        |             |             |             |
ACG GTA CCG GTG GAC TTG GCA TCA AGG CAG GAA GAA GAG GAG CAG
Thr Val Pro Val Asp Leu Ala Ser Arg Gln Glu Glu Glu Glu Gln
283

3500          3510          3520          3530          3540
  |             |             |             |             |
TCG CCC GAC TCC ACA GAG GAA GAA CCA GTG ACT CTC CCA AGG CGC
Ser Pro Asp Ser Thr Glu Glu Glu Pro Val Thr Leu Pro Arg Arg
298



      3550          3560          3570          3580
        |             |             |             |
ACC ACC AAT GAT GGA TTC CAC CTG TTA AAG GCA GGA GGG TCA TGC
Thr Thr Asn Asp Gly Phe His Leu Leu Lys Ala Gly Gly Ser Cys
313

3590          3600          3610          3620          3630
  |             |             |             |             |
TTT GCT CTA ATT TCA GGA ACT GCT AAC CAG GTA AAG TGC TAT CGC
Phe Ala Leu Ile Ser Gly Thr Ala Asn Gln Val Lys Cys Tyr Arg
328

      3640          3650          3660          3670
        |             |             |             |
TTT CGG GTG AAA AAG AAC CAT AGA CAT CGC TAC GAG AAC TGC ACC
Phe Arg Val Lys Lys Asn His Arg His Arg Tyr Glu Asn Cys Thr
343
```

Fig. 20

```
3680          3690          3700          3710          3720
  |             |             |             |             |
ACC ACC TGG TTC ACA GTT GCT GAC AAC GGT GCT GAA AGA CAA GGA
Thr Thr Trp Phe Thr Val Ala Asp Asn Gly Ala Glu Arg Gln Gly
358

      3730          3740          3750          3760
        |             |             |             |
CAA GCA CAA ATA CTG ATC ACC TTT GGA TCG CCA AGT CAA AGG CAA
Gln Ala Gln Ile Leu Ile Thr Phe Gly Ser Pro Ser Gln Arg Gln
373

3770          3780          3790          3800          3810
  |             |             |             |             |
GAC TTT CTG AAA CAT GTA CCA CTA CCT CCT GGA ATG AAC ATT TCC
Asp Phe Leu Lys His Val Pro Leu Pro Pro Gly MET Asn Ile Ser
388

      3820          3830          3840          3850
        |             |             |             |
GGC TTT ACA GCC AGC TTG GAC TTC TGA TCA CTG CCA TTG CCT TTT
Gly Phe Thr Ala Ser Leu Asp Phe --- Ser Leu Pro Leu Pro Phe
403
```

Fig. 20

```
                10        20        30        40        50        60
                 |         |         |         |         |         |
7441 TTTTCACAC ATAGCGGGAC CGAACACGTT ATAAGTATCG ATTAGGTCTA TTTTTGTCTC
7501 TCTGTCGGAA CCAGAACTGG TAAAAGTTTC CATTGCGTCT GGGCTTGTCT ATCATTGCGT
7561 CTCTATGGTT TTTGGAGGAT TAGACGGGGC CACCAGTAAT GGTGCATAGC GGATGTCTGT
7621 ACCGCCATCG GTGCACCGAT ATAGGTTTGG GGCTCCCCAA GGGACTGCTG GGATGACAGC
7681 TTCATATTAT ATTGAATGGG CGCATAATCA GCTTAATTGG TGAGGACAAG CTACAAGTTG
7741 TAACCTGATC TCCACAAAGT ACGTTGCCGG TCGGGGTCAA ACCGTCTTCG GTGCTCGAAA
7801 CCGCCTTAAA CTACAGACAG GTCCCAGCCA AGTAGGCGGA TCAAAACCTC AAAAAGGCGG
7861 GAGCCAATCA AAATGCAGCA TTATATTTTA AGCTCACCGA AACCGGTAAG TAAAGACTAT
7921 GTATTTTTTC CCAGTGAATA ATTGTT //
   1 GTTAACAATA ATCACACCAT CACCGTTTTT TCAAGCGGGA AAAAATAGCC AGCTAACTAT
  61 AAAAAGCTGC TGACAGACCC CGGTTTTCAC ATGGACCTGA AACCTTTTGC AAGAACCAAT
 121 CCATTCTCAG GGTTGGATTG TCTGTGGTGC AGAGAGCCTC TTACAGAAGT
```

Fig. 21

Self-replicating vector for DNA immunization against HIV

Field of the invention

The invention is directed to a self-replicating recombinant vector useful in DNA immunization against HIV. The invention is also directed to a vaccine comprising said vector, a method for preparing the vector, and a host cell comprising it. The invention further relates to the use of said vectors for the manufacture of a vaccine against HIV and to a method of treating or preventing HIV.

Background of the invention

The interaction of vertebrates and thus also human beings with pathogenic microbes, such as bacteria, fungi and viruses is regulated by the capacity of the vertebrate organism to mount an immune response towards the invading microbe. This reaction is based on the capacity of immune system to distinguish between self and non-self; in normal situation immune response tolerates the own structures, cells and antigenic molecules of the organism while attacks foreign antigens, expressed by the invading microbes. When a vertebrate, such as man, is infected with microbes, the immune response helps in clearing the infection by killing microbes or cells infected with microbes or by preventing the spread of infection through the action of neutralizing antibodies. Secondly, and more importantly, immune response once elicited to an invading organism has an inborn mechanism of memory and thus an individual who has once experienced infection with a particular microbe is often immune and can not be infected the second time.

This immunological memory, caused in natural situation by infection is the basis for vaccines that mimic natural infection in many ways. An ideal vaccine will cause no or only slight symptoms in the vaccinated individuals but still result in induction of immunological memory with a capability to mount a strong preventive immune responsive in case the vaccine is encountered with the microbe in question.

The design of a vaccine against a particulate microbe is dependent on the mechanism by which organism in a natural infection may clear this particular organism and prevent subsequent infections. There are several ways how immune response can elicit its favorable function. Firstly, antibodies synthesized and secreted by the B lymphocytes can bind to microbe and through complement-mediated lysis destroy it. Secondly, neutralizing

antibodies can prevent a spreading of infection by inhibiting with the binding of the microbe to its target cell. Thirdly, antibodies in conjunction with complement activation can destroy infected cells, and, finally, specific cytotoxic T lymphocytes (CTL) can kill and destroy cells infected with the microbe. All these mechanisms have been thought to be involved in the immune response elicited by the human immunodeficiency virus (HIV) type 1 and 2 (HIV-1 and HIV-2). However, the immune response in HIV-infected individuals is usually characterized by a strong antibody response and less effective or even lacking T lymphocyte response (Gerstott, J. et al. Scand. J. Immunol. 22(5):463-470, 1985; Re, M.C. et al. J.Clinical Pathol. 42(5):282-283, 1989). This may be the reason why individuals, once infected with HIV developed a chronic infection despite the strong antibody-mediated immune response.

Preventive immune response towards viral infection in general can be mediated by the four types of immune response described above, but it is known that the CTL response, capable of killing viral infected cells is most effective. This is the reason why generally speaking live attenuated viral vaccines have proven to be most effective. In fact, the first vaccine developed by Jenner more than 200 years ago, the live vaccinia virus that can prevent the infection by the small-pox virus is an example of this principle. When an individual is vaccinated with a live attenuated viral vaccine, host cells are infected, viral proteins are synthesized. Some of the viral protein molecules are used to produce virus particles while others are proteolytically cleaved to small peptides that bind to the major histocompatibility (MHC) antigens (in man HLA class I and II) and are presented to T lymphocytes on the surface of infected cells. Subsequently, T lymphocytes carrying a proper T cell receptor (TCR) will recognize the foreign peptide in association with HLA and either give help to B cells for antibody production (helper/inducer T cells; Th) or destroy the infected cell (cytotoxic T lymphocyte; CTL).

In spite of a decade of efforts, an effective vaccine against human immunodeficiency virus (HIV) infection and AIDS is still lacking. Most earlier efforts have concentrated on obtaining sterilizing immunity with neutralizing antibodies against the outer envelope glycoprotein of HIV, gp120/gp160. Phase I/II studies with gp160/gp120 demonstrate, however, the neutralization against laboratory strains only but failure to neutralize field isolates.

In contrast, experiments with attenuated virus have been successful in the simian immunodeficiency (SIV) model. SIV deleted in the NEF or REV

gene behaves as an attenuated virus and protects the vaccinated animals against disease development but not against infection by the wild-type challenge virus. With a REV- defective virus, the only immunological correlate with protection was cell-mediated immune (CMI) response against SIV regulatory proteins NEF and TAT. An important observation in this experiment was, that vaccinated animals could even clear the infection caused by the wild-type challenge virus. It has recently been reported that also HIV-infected patients may occasionally clear an overt infection. The only relevant correlation to protection in these animal and/or human studies seems to be a cell mediated immune response towards HIV.

This type of immune response is generally not obtained with protein immunization. In terms of HIV, live attenuated vaccines could be effective to prevent infection but they are theoretically dangerous for several reasons. A recently described method, genetic immunization (synonyms: nucleic acid immunization, DNA immunization) has several of the advantages of live attenuated vaccines but not their potentially harmful adverse effects. A DNA vaccine, in a form of eukaryotic expression vector that carries the gene for one or a few of the viral proteins can induce the synthesis of the viral protein, once the DNA vector is transfected into host cell. Viral proteins synthesized in the target cell will then be processed by proteolytic enzymes; the formed peptides will be bound to MHC/HLA molecules and presented on the surface of transfected cells. This will cause a CTL-mediated immunological memory that in case the individual is subsequently infected with the virulent wild-type virus will be effective in killing viral infected cells immediately upon infection and thus preventing the infection. Direct intramuscular or intradermal injection of cDNA in an eukaryotic expression plasmid has been shown to induce an immune response (Wolff et al. Science 246:1465-1468, 1990). The expression of foreign antigens by such means results predominantly in helper T-cell subset 1 (TH1) type immune responses, with strong cytotoxic T-lymphocyte (CTL) response and, occasionally, also high- titer antibody response (Wang, B. et al, PNAS 90: 4156-4160. 1993; Wang et al. Ann. NY Acad. Sci 772: 186-197, 1995; Haynes et al. AIDS Res. Hum. Retroviruses 10 (2):43-45, 1994). Moreover, using viral nucleoprotein antigen of influenza A, antigen-specific CTL and protection has been reported (Ulmer. et al. Science 259, 1745-1749, 1993). Furthermore, protection against infection using DNA immunization has been obtained for mycoplasma in mice (Barry et al.

Nature 377(6550):632-635, 1995; Lai et al. DNA Cell. Biol. 14(7):643-651, 1995) and for human papillomavirus in a rabbit model (Donnelly et al. J. Inf. Dis. 173(2):314-320, 1996). DNA immunization has also been used to induce antitumor immunity mediated by cytotoxic lymphocytes (Bohm et al. Cancer Immunol. Immunother. 44(4):230-238, 1997).

DNA immunization has several advantages in comparison to live attenuated viral vaccines. As no infectious virus is formed, the viral genes induced to the host organism stay only in those cells that are originally transfected and no symptoms of virus infection occurs. In question of HIV, the major theoretical harmful effect for a live attenuated virus would be reversion, by mutations to a virulent wild-type virus. Furthermore, with DNA immunization only those viral genes, or parts of thereof that are known to be effective in inducing preventive immune response can be used.

For an effective CTL response, it would be important that the cytotoxic T-cells would destroy the infected cells before structural proteins are formed and prior to the release of mature viral particles. Therefore, immune response towards the early proteins in viral cycle would be beneficial. The replication of HIV is regulated by its own regulatory genes and proteins. The HIV genome encodes three nonstructural regulatory proteins (NEF, TAT, REV) which are indispensable for the replication of the virus in vivo. REV is a transporter of genomic RNA into the cytoplasm, TAT upregulates viral transcription and NEF provides replication in resting cells. Experiments with SIV indicate that viruses lacking the function of one of these regulatory genes may not be able to induce disease because of insufficient viral replication. These three proteins are expressed transiently and in small quantities during the first hours of the viral infectious cycle (Ranki et al. Arch.Virol. 139:365-378, 1994). Only a small proportion of HIV-infected individuals shows humoral and/or cellular response to these proteins, and the response correlates with a favorable clinical course.

CTL responses against NEF, TAT and REV have been extensively studied. NEF-specific CTL and Th (T helper cell) responses correlate with a favorable clinical prognosis. With a REV defective SIV-vaccine, immune responses to NEF and TAT were protective. Th and CTL epitopes in TAT and REV proteins, which are recognized by HIV-1 infected individuals and which show a clinical correlation, have been identified (Blazevic et al. J AIDS 6:881-890, 1993; Blazevic et al. AIDS Res. Hum. Retroviruses 11:1335-1341, 1995).

Taken together, these results indicate that for protection against disease a moderate replication of virus (attenuated growth) in combination with specific immune responses against the regulatory proteins involved in support of virus replication may be necessary.

Several eukaryotic expression vectors can be used in DNA immunization but their efficacy varies. Some of the parameters that regulate the efficacy of a given expression vector in inducing the immune response are unknown but obviously high level of expression of the antigenic protein would be advantageous. The time period that the vector, introduced to the cell can express the foreign antigenic viral protein may also be of importance. Finally, expression vectors that induce certain level of cell injury may also be advantageous as it is known that tissue destruction will amplify immune response through several biologically active molecules, such as cytokines, lymphokines and chemokines, secreted by the cell expressing the antigenic protein. This is probably one further reason why live attenuated virus that causes a certain level of tissue and cell destruction is so effective in inducing immunity, and thus a DNA vector that in this respect mimics live attenuated vaccine would be advantageous.

Nonspecific factors such as cytokines and lymphokines may also regulate the viral replication and immune responses in HIV-1 infection. The role of the helper cell Th1/Th2 balance, reflected by production of lymphokines specific for the two helper T-cell populations, has been demonstrated by Clerici and Shearer (Immunology Today 14(3):107-111, 1993) and others. Soluble factors, produced by CD8 cells and capable in suppressing the viral production by HIV-1 infected CD4 cells, were recently identified as RANTES, MIP1-α and MIP1-β (Cocchi et al. Science 270(5243):1811-1815, 1995). It is possible, that cytokines whose production is either increased or decreased in HIV-1 infection will regulate viral transcription.

Previous studies on DNA immunization using the gene encoding the HIV regulatory protein NEF have demonstrated T-cell proliferative responses (Hinkula et al. Vaccine 15 (8):874-878, 1997 and Hinkula et al. J. Virol. Jul, 71(7):5528-5539, 1997). However, it is the CTL response that has a positive effect correlation with a favorable clinical course.

One of the main objects of the present invention is therefore to provide a DNA immunization vaccine encoding an HIV regulatory protein, the vaccine being capable of eliciting a CTL response against HIV infected cells in

the early phase of the infectious cycle, before new mature infectious viral particles are released.

Another object of the invention is to provide a vaccine, which further elicits a humoral response against HIV.

A further object of the invention is to provide an HIV vaccine, which is safe to use, because it does not expose the recipient to the structural genes or proteins of HIV.

Another object of the present invention is to provide a self-replicating vector that causes a prolonged and high level of HIV regulatory protein expression and a certain degree of cell destruction, which will further stimulate the immune response.

Still another object of the invention is to provide a self-replicating recombinant vector expressing HIV regulatory proteins, which vector confers long-term stable maintenance and a high copy number in transfected cells including mammalian cells.

A further object of the invention is to provide a host cell comprising said vector.

Yet another object of the invention is to provide a method for preparing the above-mentioned self-replicating vector.

The present invention further provides a method of treating or preventing HIV.

Still another object of the invention is the use of said vector for the manufacture of a DNA immunization vaccine against HIV.

Summary of the Invention

The objects of the present invention can be achieved by incorporating a heterologous nucleotide sequence encoding the HIV regulatory protein NEF, REV or TAT or an immunologically active fragment thereof into a vector comprising a papilloma virus E1 gene and E2 gene, a minimal origin of replication of a papilloma virus and a minichromosomal maintenance element of a papilloma virus.

In other words the invention is directed to a self-replicating recombinant vector comprising papilloma virus nucleotide sequences consisting essentially of

(i) a papilloma E1 gene and E2 gene,

(ii) a minimal origin of replication of a papilloma virus

(iii) a minichromosomal maintenance element of a papilloma virus, and

a heterologous nucleotide sequence encoding the HIV regulatory protein NEF, REV or TAT or an immunologically active fragment thereof.

The invention further provides a vaccine for DNA immunization against HIV comprising said vector, the use of said vector for the manufacture of a vaccine against HIV, and a method of treating or preventing HIV comprising administering to a person in need thereof an effective amount of the self-replicating vector and expressing the NEF, REV or TAT protein or an immunologically active fragment thereof in said person.

The invention still provides a method for preparing a self-replicating recombinant vector, said method comprising

A) inserting a heterologous nucleotide sequence encoding the HIV regulatory protein NEF, REV or TAT or an immunologically active fragment thereof into a vector comprising papilloma virus nucleotide sequences consisting essentially of

- (i) a papilloma E1 gene and E2 gene,
- (ii) a minimal origin of replication of a papilloma virus, and
- (iii) a minichromosomal maintenance element of a papilloma virus, and

B) transforming a host cell with the resulting self-replicating recombinant vector,

C) culturing the host cell, and

D) recovering said vector.

The invention also provides a host cell comprising said vector.

Brief description of the Drawings

Figure 1A shows the shuttle vector pUE83.

Figure 1B shows the shuttle vector pNP177.

Figure 2 shows the pBNtkREV plasmid of the invention.

Figure 3 shows the pBNsrαTAT plasmid of the invention.

Figure 4 shows the pBNsrαNEF plasmid of the invention.

Figure 5 shows the NEF expression in COS-7 cells transfected with pBNsrαNEF. The Western blot samples are taken 72 h post transfection and visualized with ECL.

Figure 6 demonstrates anti-NEF antibodies in sera of mice immunized with pBNsrαNEF as detected in Western blot. Samples 1 - 4 were

taken 2 weeks post last immunization and samples 5 - 8 were taken 4 weeks post last immunization.

Figure 7 shows CTL responses in mice immunized with the pBNsrαNEF vector. Figure 7A shows CTL responses, expressed as % specific lysis of the target cells, in the four mice tested two weeks after the last immunization. Figure 7B shows the values at four weeks after the last immunization. Specific lysis > 4 % is considered positive.

Figure 8 shows the immunoglobulin subclass distribution in three mice immunized with pBN-Nef.

Detailed description of the invention

According to the invention the heterologous HIV nucleotide sequence is inserted into a vector comprising a papilloma virus E1 gene and E2 gene, a minimal origin of replication of a papilloma virus (MO), and a minichromosomal maintenance element of a papilloma virus (MME). This E1/E2/MO/MME comprising vector is hereinafter called pBN, and it has been described in detail in WO 97/24451, which is incorporated by reference. Said patent publication is based on the discovery that DNA replication in papilloma viruses from the MO per se is not sufficient for stable long-term persistence, but in addition another viral sequence MME is required and that the best results are obtained when the vector further comprises the E1 and E2 genes of the papilloma virus.

'Papilloma virus' as used herein means any member of the papilloma virus family. Preferably the papilloma virus used in the invention is bovine papilloma virus (BPV) or human papilloma virus (HPV).

'E1' and 'E2' are regulatory proteins of papilloma viruses, which replicate via MO and which are necessary for replication.

'Minimal origin of replication' (MO) is a minimal sequence of a papilloma virus which is necessary for initiation of DNA synthesis.

'Minichromosomal maintenance element (MME) refers to a region of the papilloma viral genome to which viral or human proteins essential for papilloma viral replication bind. MME is essential for stable episomal maintenance of the papilloma viral MO in a host. Preferably MME comprises multiple binding sites for the transcriptional activator protein E2.

'Self-replicating vector' as used in the present application means a vector plasmid capable of autonomous replication in a eukaryotic host cell.

'Heterologous' means foreign. For example with respect to the vectors of the invention a heterologous nucleotide sequence means a non-papilloma sequence.

'Immunologically active fragment' means a fragment capable of eliciting an immunological response in a recipient.

'Papilloma virus nucleotide sequences consisting essentially of" means that the vector comprises the papilloma nucleotide sequences which are necessary and sufficient for long-term vector persistence and replication. This means for example that superfluous sequences like all papilloma-encoded oncogenic sequences have been deleted from the pBN vectors used in the present invention.

In addition to the E1 and E2 genes, MO, MME and the NEF, REV or TAT gene, the vectors of the invention comprise promotors for the encoded proteins as well as additional regulatory sequences, poly-adenylation sequences and introns. Preferably the vectors also include a bacterial host cell origin of replication and one or more genes for selectable markers for the preparation of the vector DNA in a bacterial host cell.

An essential feature of the pBN vectors is that they are not host cell specific. This is because the expression of the E1 and E2 proteins is controlled by promotors which are non-native i.e. heterologous. Said promotors are either functional in a broad range of mammalian cells or tissues or are cell- or tissue-specific.

In the vectors of the present invention the E1 gene is preferably under the control of the sr-α promotor or the thymidine kinase promotor (tk) and the E2 gene is preferably under the control of the LTR gag promotor. The NEF, REV or TAT gene can be under the control of a CMV promotor or an RSV LTR promotor. The vector can further comprise an SV40 early promotor to induce the expression of the gene for antibiotic selection (neomycin or kanamycin).

The host cell origin of replication in the vectors of the invention is preferably pUC ORI and the selective markers used are e.g. kanamycin and/or neomycin. Preferably the intron is the beta-globin IVS.

The octseq found in TK-promoter based plasmids is a non-coding sequence from octamer protein. It has no functional purpose in the plasmid, but was needed for creating suitable restriction sites for the preparation of the final plasmids.

The NEF, REV or TAT genes to be inserted into pBN can be obtained from several commercial sources such as the plasmid pKP59, which is available from the AIDS Reagent Project MHC repositary. Said genes are well known and have been fully sequenced (Wain-Hobson, et al. Cell 40:9-17, 1985). Of course it is also possible to insert a sequence encoding only an immunologically active fragment of said HIV proteins.

The NEF, REV or TAT genes or their fragments are first inserted into appropriate shuttle vectors. These vectors can either include or not include the MO region. Two shuttle vectors are illustrated in the examples: pNp177, which does not include the MO, and pUE83, which includes the MO. Of course it is possible to use other shuttle vectors too. Both the shuttle vectors and the resulting vectors of the invention are preferably multiplied in Escherichia coli. Examples of the resulting pBN-NEF, pBN-REV or pBN-TAT vectors of the present invention are set forth in Figures 2, 3 and 4. The vectors of the invention are stable and self-replicating in a large copy number. Upon transfection into a eukaryotic host cell, the vector (plasmid) will multiply and produce 100 - 1000 fold amount of new plasmids, each capable of expressing the HIV protein in demand.

The host cell claimed in the present invention can be either a eukaryotic cell transfected by the vector or a prokaryotic cell transformed by the vector. The eukaryotic cell is preferably a mammalian cell and the prokaryotic cell is preferably a bacterial cell, especially E. coli.

The expression of HIV NEF, REV and TAT of the resulting plasmid vectors of the present invention was tested both in transfected COS-7 cells and in mice immunized with said plasmids. A high expression of the HIV proteins could be demonstrated in the COS-7 cells and the immunized mice showed a remarkable humoral and cell mediated (CTL) immune response. A significant CTL response was also demonstrated in monkeys. These results indicate that the vectors of the invention have a potential use as effective vaccines against HIV.

It was further demonstrated that a mixture of pBN-vectors encoding different HIV reagulatory proteins mounted an immune response to several regulatory genes. The present invention thus includes vaccines comprising a mixture of vectors encoding different HIV regulatory proteins or immunologically active fragments thereof and the use of said mixture in the manufacture of the vaccine and the treatment or prevention of HIV. The vaccine

may contain a mixture of vectors encoding all three different regulatory proteins. The vaccines of the present invention may also contain other genes or gene fragments e.g. selected from the group consisting of the HIV structural genes.

The present invention is further illustrated in the following examples. The examples describe in detail some embodiments of the invention, but they should not be interpreted to restrict the invention, which is defined by the attached claims.

Example 1

Cloning of HIV-1 genes REV and TAT into self-replicating pBNsr-α and pBNtk plasmids

Production of pBNtkREV and pBNsraTAT

Phase 1:

Sub. B1

The HIV-1 REV and TAT genes from isolate BRU also called LAI (Wain-Hobson et al. Cell 40:9-17, 1985) were amplified from the pcREV and pcTAT vectors (Arya et al. Science 229:69-73, 1985) using Dynazyme Taq DNA polymerase (Finnzymes, Finland) and the following primers that have restriction enzyme sites for enzymes XhoI and XbaI:

For REV:

Sub. B2

5´-TTTTTCTAGAACCATGGCAGGAAGAAGCGGA-3´

5´-TTTTCTCGAGCTATTCTTTAGTTCCTGG-3´

For TAT:

Sub. B3

5´-TTTTCTAGAACCATGGAGCCAGTAGATCCT-3´

5´-TTTTCTCGAGCTAATCGAACCGGATCTGC-3´

The amplified genes and pUE83 shuttle vector (Figure 1) were digested at +37 °C with XbaI and XhoI (New England BioLabs, USA) overnight in order to get compatible ends. The digested DNA-fragments were analyzed on 1.5 % agarose gels, and further purified using Band Prep Kit (Pharmacia Biotech, Sweden). Each gene was ligated into the vector separately using T4 DNA ligase (New England BioLabs, USA) in an overnight incubation at +16 °C. The ligation products were transformed into One Shot Kit (Invitrogen, The Netherlands) competent E. coli cells, which were plated on LB-plates containing kanamycin for selection. Minipreps were prepared from the growing

clones, and the presence of cloned genes was analyzed by digestion with XhoI and XbaI. The presence of the cloned genes was also confirmed by PCR from miniprep preparation using the above mentioned primers. Clones containing the right gene were mass cultivated and plasmids were purified using Megaprep columns (Qiagen, Germany).

Phase 2:

A DNA fragment containing BPVori, RSV LTR promoter, REV- or TAT-gene and b-globin IVS poly(A) was digested from the shuttle vector by HindIII (New England BioLabs, USA), and purified using 1 % agarose gel and Band Prep Kit. Ligation to HindIII digested and dephosporylated (alkaline phosphatase, CIP, Promega, USA) pBNsrα or pBNtk, transformation of cells, verification of the presence of cloned gene and purification of the plasmid were done as in phase 1.

The resulting plasmids are called pBNtkREV and pBNsrαTAT and are set forth in Figures 2 and 3.

Example 2

Cloning of HIV-1 NEF into self-replicating pBNsrα plasmid

Production of pBNsrαNEF

~~Phase 1:~~

Sub. B4

The HIV-1 NEF gene was obtained from a plasmid pcNEF vector, which contained the LAI isolate NEF gene inserted into a pcTAT vector lacking the TAT gene. The NEF gene used for further cloning was achieved as a 1.3 kb fragment by Spe I and Hind III digestion from pcNEF. To eliminate the reformation of the Hind III site on ligation, after Hind III digestion the fragment was treated with Klenow enzyme and a mix of dATP, dCTP, dGTP nucleotides after which the Spe I digestion was performed. The fragments obtained were separated by electrophoresis on a 1% agarose gel alongside standard size markers. Bands of correct size were cut out and the DNA recovered using the Sephaglas Bandprep Kit (Pharmacia Biotech), following the manufacturer's protocol.

The shuttle vector pNP177 of Figure 1B was first digested with Xho I, then treated with Klenow enzyme and dNTP mix, and, finally, digested with Xba I. The vector was also treated with calf intestinal alkaline phosphatase (CIP).

The fragment containing the NEF gene was ligated with cleaved pNP177 vector by using T4 ligase in +14°C overnight. One Shot competent E.

coli kit (Invitrogen) was used for transformation. Positive clones were identified by using restriction enzyme digestions and electrophoresis. Plasmid DNA was further amplified in E. coli and purified in a large scale with Qiagen columns. The resulting final plasmid was called pNP177cHIVNEF.

Phase 2:

The shuttle vector pNP177 is designed to have only two Hind III sites between which an insert can be cloned. A Hind III digest of the plasmid thus gives a fragment which can be cloned further. The Hind III fragment of pNP177cHIVNEF was cloned into pBNsrα. The vector was digested with Hind III and treated with CIP. The same methods of band separation, ligation, transformation were used as in the first phase and correct orientation of the insert was confirmed by restriction analysis. The final plasmid was called pBNsrαNEF and is shown i Figure 4.

Example 3

Demonstration of expression of HIV-Nef in vitro

3A.Transfections

To test the expression of the pBN-constructs of examples 1 and 2, they were transfected by electroporation into COS-7 cells. 10 μg of pBNβ-Gal as a control, and 10 μg of pBN-NEF cotransfected with 1μg pCMVβ-Gal, were electroporated each into three million cells. Salmon sperm carrier DNA was used. The electroporation was made at 960 mF capacitance and 260 V voltage. Protein concentration and β-gal activity measurements were made to control the efficiency of transfection and to calibrate the amount of the lysates in Western blot assay.

3B. Immunohistochemistry and Western blotting

The harvested cells transfected with the pBN-constructs were lysed for use in Western blotting. After lysis, protein samples were boiled in sample buffer and run in a 12% SDS polyacrylamide gel, then transferred onto a 2 μm nitrocellulose filter which was blocked with a solution of 5% milk in TBS. As a primary antibody a mixture of mouse anti-NEF monoclonals (Ovod V. et al. AIDS 6:25-34, 1992) diluted to 1:1000 each was used. The secondary antibody was a biotinylated anti-mouse in a 1:500 dilution.

After transfection to COS-7 cells, the vectors produced a strong transient HIV regulatory protein expression, as detected by Western blotting of the lysed cells at 72 hours. The results obtained with pBNsrαNEF are shown in Figure 5. In long-term cultures of the transfected cells, NEF expression

sustained up to 7 weeks in the cells transfected with the self-replicating pBN vector.

The NEF-transfected cells were also used to prepare cytospin preparates and they were stained with haematoxylin and a monoclonal antibody against NEF followed by a secondary biotinylated anti- mouse were used in immunohistochemistry as described in Ovod et al. supra. The cytospin slides indicated expression as positive staining was seen in a large number of cells as granules occupying the cell cytoplasm. A portion of the NEF expressing cells showed morphological signs of cell destruction, indicating apoptosis. Still the level of expression was high though the condition of the cells was getting worse.

Example 4.

Demonstration of immunogenicity of pBNsrα and pBNtk vector expression of HIV-Nef, HIV-Tat or HIV-Rev in vivo

4A. Gene Gun

DNA was precipitated onto 1 μm gold particles using spermidine and $CaCl_2$ following the procedure in the Helios Gene Gun Instruction Manual (Bio-Rad Laboratories). Cartridges were made to carry 0.5 mg gold and 1 μg DNA each. The amount of DNA was controlled spectophotometrically as instructed in the manual. Inoculations were performed using the Helios Gene Gun System (Bio-Rad Laboratories). Helium discharge pressure for DNA delivery was set to 300 psi. In our optimization of the bombardment conditions we found 300 psi to be sufficient to propel the gold particles into the dermis.

4B. Immunizations

Female 6-8 week-old balb/c mice were used. Before immunizations the mice were anesthetized and the abdominal fur was removed.

Inoculations on the abdominal skin of 8 mice were done on days 1, 2, 3, 10, 11 and 12 using the gene gun described above and following the instruction of the manufacturer. A total of 6 μg of pBN-NEF was administered per mouse. Four mice from both groups were sacrificed two weeks post last immunization and the remaining four mice four weeks post last immunization. Serum samples for Western blotting were taken and splenocytes harvested for a CTL assay. All eight mice immunized with the pBN-NEF- vector, showed an antibody response at 2 weeks and 4 weeks (Figure 6). The intensity of the reaction in the Western blotting varied.

4C. Measurement of cytotoxic T-cell activity in the immunized mice

4C1. Stimulation of effector cells

Spleens were removed aseptically from the immunized mice two (16 mice) and four weeks (16 mice) after immunization. They were disrupted in Hanks, filtered through gauze and the erythrocytes were removed. Cells were then suspended 5 x 106 / ml in culture medium: RPMI 1640 medium containing 10 % fettle calf serum (FCS; GibcoBRL), 1 % glutamin, 100 U of penicillin per ml, 100 (g of streptomycin per ml and 5 x 10-5 M 2-mercaptoethanol. The responding cells (5 x 106) were co-cultured in 25 ml cell culture flask in 5 ml of culture medium with 4 x 106 antigen presenting cells (APCs; see below) for five days. 10 U / ml of recombinant interleukin - 2 (rIL-2) was added at the first day to the cells (Hiserodt J. et al. J. Immunol. Jul, 135(1):53-59, 1985; Lagranderie M. et al. J. Virol. Mar, 71(3):2303-2309, 1997; Tsuji T. et al. Immunology Jan. 90(1):1-6, 1997; Vahlsing H. L. et al. Journal of Immunological Methods 175:11-22, 1994; Varkila K. et al. Acta path. Micorbiol. Immunol. Scand. Sect. C 95:141-148, 1987)

4C2. Antigen presenting cells

Syngeneic P815 mastocytoma (H-2d) cells were infected with modified vaccinia virus Ankara (MVA) engineered to express the HIV-1 LAI NEF gene (MVA-HIVNEF). MVA is a highly attenuated replication-deficient vaccinia virus, which can serve as an efficient vector for expression of heterologous genes providing an exceptionally high level of biological safety (Sutter G. et al. J. Virol. Jul, 68(7):4109-4116, 1994; Sutter et al. Vaccine 12(11):1032-1039, 1994; Drexler I. et al. J. Gen. Virol. 79:347-352, 1998; Sutter G. et al. Proc. Natl. Acad. Sci. USA 89:10847-10851, 1992). Infections with MVA-HIVNEF were performed at a multiplicity of infection (MOI) of 5 in 24 - well plates (1 x 10^6 cells per well). After 1h virus absorption at +37°C, the cells were incubated for 15 h in +37°C (Carmichael A. et al. Journal of Virology 70:8468-8476, 1996). After infection the cells were washed twice with PBS (phosphate buffered saline) containing 10 % FCS and suspended in this solution 5 x 10^6 cells/ml. Cells were then γ-irradiated at 5000 rad and washed with culture medium before adding to responder cells.

4C3. Cytotoxicity assays

CTL activity was tested by the ^{51}Cr- release assay (Hiserodt J. et al. J. Immunol. Jul, 135(1):53-59, 1985; Lagranderie M. et al. J. Virol. Mar,

71(3):2303-2309, 1997; Varkila K. et al. Acta path. Micorbiol. Immunol. Scand. Sect. C 95:141-148, 1987; van Baalen C. et al. AIDS 7:781-786, 1993). Briefly, 2 x 10^6 P-815 cells were infected with MVA-HIVNEF as described above for antigen presenting cells. After infection the cells were washed once in serum free culture medium. Target cells then were suspended in 200 µl of serum free culture medium and 100 µCi of ^{51}Cr (Amersham) / 1 x 10^6 cells was added for 1 h at 37°C. Target cells were then washed four times in medium and suspended in concentration 5 x 10^4 / ml. The stimulated effector cells were washed once in culture medium before adding to the target cells. Target cells were plated in u-bottom 96- well plate 100 µl (5 x 10^3) per well and effector cells were added in triplicates in 100 µl at effector: target ratios 50, 25 and 12.5. For spontaneous release, target cells were plated in six wells with 100 µl of culture medium and for maximum release in six wells with 2.5 % Triton-X-100. The plates were spun briefly, incubated for 4 hours in 37°C and the supernatants were counted in a gamma-counter. The percent specific lysis of target cells was calculated as (test ^{51}Cr release - spontaneous release)/(maximum release - spontaneous release) x 100. The percent specific lysis ≥ 6 % was considered to be positive.

An example showing CTC activity in 6 of the 8 mice immunized with pBNsrαNEF is shown in figure 7.

D. Humoral immune response in immunized mice

To test the occurrence of antibodies against HIV-1 NEF in immunized mice sera NEF protein was electrophoresed on PAGE, transferred to nitrocellulose filters and the antibody reactivity was detected as described above.

Summary of the results

The results of the transfection and immunization tests are summarized in Table 1. The immune response in the immunized mice was assessed by immunoblotting (WB) for humoral and by cytotoxic t-lymphocyte (CTL) assay for the cell mediated immunity. As seen in the table, all eight mice immunized with the NEF expressing vector showed both humoral and cell mediated immune response.

Table 1. Demonstration by immunoblotting (Western blotting, WB) or by immunohistochemistry of the expression of the HIV-1 NEF, TAT and REV proteins in COS-7 cells transfected with said vectors and demonstration of induction of humoral and cell mediated immune response in mice immunized with one of the vectors, pBNsrαNEF

	Transfection		Immunization	
	WB	Immunohisto-chemistry	WB	CTL
pBNsrαTAT	ND	++	6/8	6/8
pBNtkREV	+	++	7/8	7/8
pBNsrαNEF	++	ND	6/8	6/8

In this study we demonstrate that DNA immunization using a self-replicating expression vector as described can induce a clearly detectable CTL response in mice. In addition a humoral immune response was achieved. In view of the above results it is feasible to assume that the pBN-NEF, pBN-REV and pBN-TAT plasmids do express NEF, REV and TAT in vivo in an amount sufficient to induce both the humoral and the cell-mediated immune response necessary for preventing or treating HIV.

Example 5

Measurement of Th1/Th2 type response in intramuscularly immunized mice

The humoral immune response seen in mice immunized with pBN constructs expressing HIV regulatory proteins was tested for immunoglobulin subclass specificity. It is well known that antibody response dominated by IgG2a subclasses of immunoglobulins is a characteristic of a Th1 type cell-mediated immune response while IgG1, IgG2b and IgG3 are characteristic for a Th2 cellular response. Furthermore, Th1 type responses are known to induce and help cell-mediated cytotoxic immune responses (CTL response) while Th2 response will induce antibody response but less active CTL responses.

The immunization schedule was as follows:

Four Balb/c mice were immunized six times with 24 micrograms of pBN-Nef of Example 2 in two weeks, the total amount of DNA being thus 144

and the sera analyzed for antibodies in Western blot. Three out of four mice had antibodies against HIV-1 Nef and the subclass of these antibodies was measured in an ELISA assay as follows:

The antigen was pipetted on Nunc Maxi Sorb plates for overnight incubation in +4 °C; the antigen used was HIV-1 Nef protein (NIH, AIDS Research and Reference Reagent Program) in PBS (50 ng/well). The plates were blocked in an overnight incubation with 1 % BSA (Sigma), and thereafter incubated with the mice sera (diluted 1:100 in blocking solution) for 4 hours at room temperature. Plates were washed with PBS-0.1 % Tween 20 three times and thereafter with PBS two times. As secondary antibodies peroxidase conjugated anti-mouse IgG1, IgG2a, IgG2b, IgG3 and IgM (Calbiochem) diluted 1:1000 in blocking solution were used and the plates were incubated 2 hours at room temperature. After washing steps performed as earlier described, substrate ABTS (Sigma) and H_2O_2 in citrate buffer was added for 10 minutes and the photometric determination was carried out in an ELISA-reader at 405 nm. The results are shown in Figure 8. The highest response in the three mice was detected with IgG2a secondary antibody (Absorbance at 405 nm 0.117, 0.262, 0.743 respectively), the response with IgG1 antibody being much lower (A(405) 0.004, 0.020, 0.038). This indicated that the type of response in these mice after intramuscular immunization is merely Th1-type leading to cell mediated immune response.

The results demonstrate that practically all mice had a strong IgG2a type response toward recombinant Nef while antibodies representing other IgG subclasses were very low. The results further prove that the pBN Nef construct is able to mount a Th1 type response and subsequently strong cell-mediated immune response capable of destroying HIV-infected cells in the early phase of viral infectious cycle.

Example 6

Generation of cell-mediated immune response in Macaca fascicularis monkeys by pBN constructs expressing HIV-1 regulatory proteins

Experiments with mice clearly indicated that the pBN Nef, pBN Rev and pBN Tat constructs were able to mount a CTL response in immunized mice. Further experiment was performed with a non-human primate to prove that the constructs could be used as preventive vaccines in human beings. It is important to demonstrate that the immune response can also be generated in non-human primates that are genetically closer to man and that can be infected with a corresponding primate retrovirus SIV that is closely related to HIV-1 and HIV-2, infecting man. We therefore performed an experiment where Macaca fascicularis monkeys were immunized with the pBN constructs expressing HIV-1 regulatory proteins Nef, Rev and Tat. These were prepared as described in Examples 1 and 2. Three Macaca fascicularis monkeys were immunized with a mixture of pBN Nef, pBN Rev and pBN Tat. Three monkeys served as controls. The immunization schedule was as follows:

Monkeys were immunized with a total amount of 300 micrograms of pBN-Nef, pBN-Rev and pBN-Tat (100 micrograms of each) twice. The first immunization was given into deltoid muscle and the second (2 weeks later) was given intradermally. Cytotoxic T-lymphocyte assays was performed two months after the last immunization as described in Example 4C3. The results were as follows:

Target cell:	Immunized monkey	Control monkey
Nef	10.2 *	0 *
Tat	10.3 *	0 *
Rev	0 *	0 *

* = Percent specific lysis of the target cell expressing corresponding HIV-1 antigen

One of the three monkeys had a demonstrable CTL response against autologous B cells expressing HIV-1 Nef and Tat. The results demonstrate that not only mice but also primates can be immunized with the pBN constructs expressing the HIV regulatory proteins and the immunized animals will mount cell-mediated T cell response characterized by the presence of cytotoxic T lymphocytes that are capable of destroying HIV infected cells in the early phase of viral infectious cycle. Furthermore, the results show that the constructs can be given simultaneously as a mixture and that the presence of one construct in the mixture does not interfere with the immune response generated with another one.

Claims

1. A self-replicating recombinant vector comprising papilloma virus nucleotide sequences consisting essentially of

(i) a papilloma E1 gene and E2 gene,

(ii) a minimal origin of replication of a papilloma virus

(iii) a minichromosomal maintenance element of a papilloma virus, and

a heterologous nucleotide sequence encoding the HIV regulatory protein NEF, REV or TAT or an immunologically active fragment thereof.

2. A self-replicating vector of claim 1 wherein the papilloma virus is bovine papilloma virus (BPV).

3. A self-replicating vector of claim 1 ~~or 2~~ wherein the heterologous nucleotide sequence encodes the HIV-1 NEF protein.

4. A self-replicating vector of ~~any of the preceding claims~~ claim 1 wherein E1 is under the control of the srα promotor or the thymidine kinase promotor.

5. A self-replicating vector of claim 4 which is pBNtkREV, pBNsrαTAT or pBNsrαNEF as shown i Figure 2, 3 or 4.

6. A vaccine for DNA immunization against HIV comprising a self-replicating vector of ~~any of claims 1 - 5~~ claim 1.

7. A vaccine of claim 6 comprising a mixture of vectors encoding different HIV regulatory proteins or immunologically active fragments thereof.

8. Method for preparing a self-replicating recombinant vector of ~~any of claims 1 - 5~~ claim 1, said method comprising

A) inserting a heterologous nucleotide sequence encoding the HIV regulatory protein NEF, REV or TAT or an immunologically active fragment thereof into a vector comprising papilloma virus nucleotide sequences consisting essentially of

(i) a papilloma E1 gene and E2 gene,

(ii) a minimal origin of replication of a papilloma virus, and

(iii) a minichromosomal maintenance element of a papilloma virus, and

B) transforming a host cell with the resulting self-replicating recombinant vector,

C) culturing the host cell, and

D) recovering said vector.

9. The method of claim 8 wherein the host cell is an E. coli cell.

10. Use of a self-replicating vector of ~~any of claims 1 - 5~~ claim 1 for the manufacture of a DNA immunization vaccine against HIV.

11. The use of claim 9 in the manufacture of a vaccine comprising a mixture of vectors encoding different HIV regulatory proteins or immunologically active fragments thereof.

12. Method of treating or preventing HIV comprising administering to a person in need thereof an effective amount of a self-replicating vector of ~~any of claims 1 - 5~~ claim 1, and expressing the NEF, REV or TAT protein or an immunologically active fragment thereof in said person.

13. The method of claim 12 comprising administering a mixture of vectors encoding different HIV regulatory proteins or immunologically active fragments thereof.

14. A host cell comprising the self-replicating vector of ~~any of claims 1 - 5.~~ claim 1

15. The host cell of claim 14, which is a bacterial cell or a mammalian cell.

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(57) Abstract

The invention relates to a recombinant vector for stable persistence of erogenous DNA in a eukaryotic host cell, and the uses of the recombinant vector for long-term stable production of a gene product in the host cell, the vector including the minimal origin of replication of papillomavirus and the minichromosomal maintenance element of papillomavirus.

EPISOMAL VECTOR AND USES THEREOF

Field of the Invention

The invention relates in general to episomal vectors.

Background of the Invention

In lower organisms, such as prokaryotes and budding yeast, replication origins contain welldefined cis-sequences called "replicators" and interaction of these sequences with a specific initiator protein complex leads to the initiation of DNA synthesis in these cells (Jacob *et al.*, 1963; Stillman, 1994 and references therein; DePamphilis, 1993). Extrachromosomal replicators, generally, in addition to their origin function, encode functions that assure equal distribution of replicated molecules (i.e., partitioning) between daughter cells at cell division. For prokaryotic plasmids these partitioning functions are well studied and can be provided by several different mechanisms in bacterial cells (Nordström, 1990). In higher organisms, less is known about mechanisms for partitioning of extrachromosomal replicators. For artificial plasmids in yeast, chromosomal centromeres can provide this function. In metazoan cells, one well studied example of a stable extrachromosomal replicator exists - the latent origin oriP from Epstein-Barr Virus (EBV). The maintenance function of EBV requires the viral replication factor EBNA-1 and a series of binding sites for EBNA-1 termed the family of repeats (FR). A model that has been suggested for the function of the EBNA-1/FR combination is that EBNA-1 bound to FR provides physical retention of the oriP plasmids in the cell nucleus (Krysan *et al.*, 1989).

Papillomaviruses are also capable of stable extrachromosomal replication. Infection and transformation of the cells by papillomaviruses follows single hit kinetics. (Dvoretzky *et al.*, 1980). Papillomavirus genomes are maintained as multicopy nuclear plasmids in transformed cells. The viral life-cycle can be viewed as three stages (Botchan *et al.*, 1986). First, following initial entry, the papillomaviral genome is amplified in the cell nucleus, i.e., viral DNA is synthesized faster than chromosomal DNA and the copy-number is increased. The second stage represents maintenance of the viral DNA at a constant copy-number and latent phase of the viral infection is established. During the third, vegetative, stage of the viral life-cycle viral DNA amplification is initiated again, late proteins are synthesized and viral particles are assembled.

The El and E2 proteins are the only viral factors required for initiation of papillomavirus DNA replication (Ustav and Stenlund 1991; Ustav *et al.*, 1991; Yang *et al.*, 1991; Chiang *et al.*, 1992; Kuo *et al.*, 1994). A similar, if not identical, set of cellular replication factors and enzymes, in addition to viral initiator proteins, is utilized by SV40 (Tsurimoto *et al.*, 1990; Weinberg *et al.*, 1990) and BPV-1 (Müller *et al.*, 1994) at the origin of replication to initiate DNA synthesis. Analysis of the essential cis-sequences shows that the BPV-1 minimal origin (Ustav *et al.*, 1993) resembles a typical eukaryotic origin of replication (DePamphilis, 1993) and it has been suggested that this similarity could also be extended to the mechanisms of replication of all papovaviruses (Nallaseth and DePamphilis, 1994; Bonne-Andrea *et al.*, 1995). However, the ability of the papillomaviruses to persist as plasmids distinguishes papillomaviruses from other papovaviruses. It has been known for more than 10 years that BPV-1 replicates in transformed cells as a multicopy nuclear plasmid, which can persist in the tissue culture cells over long periods of time (Law *et al.*, 198 1). This indicates that papillomaviruses have efficient mechanisms for segregation, i.e., control of copy-number and partitioning, in the transformed cells.

The role of viral factors, cis-acting sequences and cellular factors in long-term persistence of papillomaviruses, which relates to the segregation functions of viral DNA, is not clearly understood. That is, the regions of the viral genome which specify copy number are not identified in the prior art; nor are the regions of the viral genome which participate with the host cell to ensure proper segregation of the viral genome during partitioning. Much more is understood with respect to the initial amplification stage of the papillomavirus life-cycle.

Bovine Papillomavirus (BPV) and Human Papillomaviruses (HPVs) persist as stably maintained plasmids in mammalian cells. Transient assays, i.e., on the order of several hours to 3-4 days, have been used to define the minimal origin of replication (MO) which is required for transient replication in BPV (Ustav et al., EMBO J, 10, 4231-4329, 199 1) and for several HPV subtypes. Two trans-acting factors encoded by BPV and HPVs, namely E1 and E2, have been identified in transient assays which are necessary to mediate replication in many cell types via MO (Ustav *et al.*, EMBO J., 10, 449-457 (1991); Ustav *et al.*, EMBO J, 10, 4231-4329, (1991); Ustav *et al.*, PNAS, 90, 898-902 (1993).) El and E2 from BPV will replicate via the BPV MO and via the MO of many HPV subtypes. (Chiang *et al.*, PNAS, 89, 5799-5803 (1992). El and E2 from HPV will replicate via

the BPV MO and via the MO of many HPV subtypes. (Chiang *et al.*, PNAS, 89, 5799-5803 (1992). Replication of plasmids containing the above elements is high level but transient in eukaryotic cells. Plasmid loss is rapid in the presence and absence of selective pressure.

The papillomavirus life cycle has been the subject of much research. Different portions of the viral genome have been tested in short-term, i.e., transient, transcription or replication assays. See, for example, Szymanski *et al.*, 1991, Jour. Virol. 11:5710; Vande Pol *et al.*, 1990, Jour. Virol 64:5420; Sowden *et al.*, 1989, Nucl. Acids Res. 17:2959; Stenlund, 1987, Science 236:1666; Sedman *et al.*, 1995, Eur. Jour. Mol. Biol. 14:6218; Haugen *et al.*, 1988, Eur. Jour. Mol. Biol. 7:4245; and Kuo *et al.*, 1994, Jour. Biol. Chem. 269:24058.

The BPV 69% transforming region has been used to introduce the rat preproinsulin gene into mouse cells (Sarver *et al.*, 1981, Mol. Cell. Biol. 6:486).

The PMS1 and PMS2 regions of BPV have been reported to "independently support" extrachromosomal replication of the Tn5 neomycin gene in cells that provide viral factors in trans. PMS-1 (plasmid maintenance sequence- 1) is localized within a 521 bp region mapping at positions 6945-7476 of the BPV genome, and PMS-2 has been localized to a 140 bp region within the putative open reading frame for the El protein, which maps at positions 1515-1655 of the BPV genome. It has been reported that recombinant plasmids carrying either of the PMS elements are unrearranged and stably maintained at a constant copy number. In addition, El, E6 and E7 are identified as candidate factors for trans regulation of the plasmid state. See Lusky *et al.*, 1984, Cell 36:391, and Lusky *et al.*, 1986, Jour. Virol. 11:729.

Woo *et al.*, W094/12629 report a vector containing a papilloma virus origin of replication, the "vector maintenance sequence" described in Lusky *et al.*, 1984, supra, a therapeutic nucleic acid, and an E2 gene sequence or an E1 /E2 chimeric gene. Woo *et al.* suggest that such a vector may be tested for stable episomal maintenance over a period of 2-30 days post-transfection. The "vector maintenance sequence" of Lusky *et al.*, 1984, which is described in Woo *et al.*, is shown herein not to be capable of providing long-term vector persistence.

Mutations in the E2 gene have a pleiotropic effect on viral gene functions, including oncogenic transformation. These effects may be the result of the requirement for E2 expression to regulate viral transcription (see DiMaio and Neary, 1989, The Genetics of bovine papillomavirus type 1 papillomaviruses and human cancer. (Ed. N. Pfister), CRC Press, Boca Raton, FL). The BPV-1 E2 protein has been shown to activate viral enhancers in trans (Spalholz *et al.*, 1985, Cell 42:183). The E2 open reading frame has been shown to encode a site-specific DNA binding protein that can bind to several sites within the E2 responsive enhancers 1 and 2 (Androphy *et al.*, 1987, Nature 325:70; Moskaluk *et al.*, 1987, Proc. Nat. Aca. Sci. 84:1215). E2 recognition sites that have been studied to date include the sequence motif ACCN6GGT, where N is any nucleotide (Hawley-Nelson *et al.*, 1988, Eur. Jour. Mol. Biol. 7:525; Hirochika *et al.*, 1988,

Genes Dev. 2:54; McBride, 1988, Eur. Jour. Mol. Biol. 7:553; Moskaluk *et al.*, 1988a, Prc. Nat. Aca. Sci. 85:1826), and it is suggested that E2 binds this palindrome as a dimer (Dostani *et al.*, 1988, Eur. Mol. Biol. Org. Jour. 7:3 807; McBride *et al.*, 1989, Proc. Nat. Aca. Sci. 86:510). Not all of these sites appear to bind E2 with the same strength. Sites having the motif ACCGN4CGGT appear to bind better than sites that deviate in the fourth and ninth bases (Hawley-Nelson *et al.*, 1988, supra; Moskaluk *et al.*, 1988b, supra). It also appears that some of the target sites for the protein have different capabilities for activation in vivo (Harrison *et al.*, 1987, Nucl. Acids. Res. 15:10267; Haugen *et al.*, 1987, Eur. Jour. Mol. Biol. 6:145; Spalholz *et al.*, 1987, Jour. Virol. 61:2128). Li *et al.* (1989, Genes & Develop. 510) analyze 17 E2 binding sites in the BPV- I genome and show that affinities for E2 vary over a 300-fold range. Li *et al.* also find that the presence of the conserved consensus ACCGN4CGGT did not necessarily guarantee that the binding site would be stronger than one with a deviant base, and suggest that the presence of this palindrome is not a sufficient parameter for predicting the strength of a binding site.

A truncated form of E2 protein exists which is defective in transcriptional activation and competent in viral replication.

Dowhanick *et al.*, 1995, Jour. Virol. 69:7791, describe an E2 deletion mutant containing residues 1-218 of the protein which is said to retain a DNA replication function, but is defective in transcriptional trans-activation. Also described are several E2 point mutants (331 and 344) which are defective in both transcriptional transactivation and DNA binding.

In addition, subsequent to Applicant's disclosure of E2 point mutants which are defective in transcriptional activation and replication competent in the subject priority document, which E2 mutants are also described in Abroi *et al.*, 1996, Jour. Virol. 70:6169, additional similar, if not identical in some instances, E2 point mutants have been identified. Ferguson and Botchan, 1996, Jour. Virol. 70:4193, describe mutations at amino acids 73 and 74 which retained replication function but failed to activate transcription". Sakai *et al.*, 1996, Jour. Virol. 70:1602, describe three point mutants (R37A, 173A, and W92A) in HPV defective for transcriptional activation but retaining wild type DNA replication activity in transient assays.

One object of the invention is to provide a recombinant vector which, by virtue of the sequences it contains, is stably maintained and thus persists long-term in mammalian cells.

Another object of the invention is to provide a recombinant episomal vector which is stabilized via regulatory sequences which are contained within a relatively small amount of DNA.

Another object of the invention is to provide a cis-acting element which confers long-term stability to a transiently replicating eukaryotic episomal plasmid.

Yet another object of the invention is to provide an episomal genetic element which replicates independently of the host cell chromosomal DNA, and is therefore not dependent upon regulatory control of replication by the host cell genome.

Another object of the invention is to provide stable and reliable plasmid copy number in both G1 and G2 stages of the cell cycle.

Yet another object of the invention is to provide a recombinant vector which is stably maintained at a constant copy number for multiple cell generations.

Another object of the invention is to provide a recombinant vector which is able to persist over a long time period in eukaryotic, particularly mammalian cells, from which can be expressed a therapeutic, prophylactic, or marker gene.

Another object of the invention is to provide a recombinant vector which is not restricted as to its ability to be maintained in a given cell type, but which is stably maintained in any one of numerous diverse mammalian cell types.

Another object of the invention is to provide a recombinant vector containing sequences of viral origin which do not confer oncogenic properties to the transfected host cell, and is therefore safe to use in humans.

Summary of the Invention

The invention is based on the discovery of a vector system which permits long-term persistence in episomal form in a mammalian cell, and in particular to the discovery of a minichromosomal maintenance element, which element confers stable persistence of extrachromosomal (i.e., episomal) DNA in mammalian host cells.

The invention encompasses a method of obtaining long-term stable production of a gene product of interest in a host cell, comprising providing a host cell containing a vector comprising (A) a minimal origin of replication of a papilloma virus, (B) a minichromosomal maintenance element of a papilloma virus, and (C) a gene encoding the gene product, wherein the vector, when present in a mammalian host cell, persists in the cell for at least about 50 cell generations in dividing cells or for at least about 8 weeks in non-dividing cells under nonselective conditions without an appreciable loss of copy number.

The invention also encompasses a method of obtaining long-term stable production of a gene product of interest in a host cell, comprising providing a host cell containing a vector comprising papillomavirus sequences consisting essentially of (A) a papillomavirus E2 gene, (B) a

minimal origin of replication of a papilloma virus,(C) a minichromosomal maintenance element of a papilloma virus, and (D) a gene encoding the gene product, wherein the vector persists in the cell for at least about 50 cell generations in dividing cells or for at least about 8 weeks in non-dividing cells under nonselective conditions without an appreciable loss of copy number.

The invention also encompasses a method of obtaining long-term stable production of a gene product of interest in a host cell, comprising providing a host cell containing a pair of vectors comprising (I) a first vector comprising papillomavirus sequences consisting essentially of (A) a papillomavirus E2 gene, (B) a minimal origin of replication of a papilloma virus, and (C) a minichromosomal maintenance element of a papilloma virus, and (II) a second vector comprising papillomavirus sequences consisting essentially of (A) a gene encoding the gene product, (B) a minimal origin of replication of a papilloma virus, and (C) a minichromosomal maintenance element of a papilloma virus, wherein the vector persists in the cell for at least about 50 cell generations in dividing cells or for at least about 8 weeks in non-dividing cells under nonselective conditions without an appreciable loss of copy number.

The invention also encompasses use of a recombinant vector for obtaining long term stable maintenance of erogenous DNA in a eukaryotic host cell wherein the recombinant vector comprises: a minimal origin of replication of a papillomavirus; a minichromosomal maintenance element of a papillomavirus; and a heterologous DNA sequence encoding an expressible gene.

Preferably, the time period over which the vector persists in the host cell under nonselective conditions without an appreciable loss of copy number is 6 weeks, and most preferably 8 weeks or 12 weeks or longer, or in terms of cell generations, 100 or 120 cell generations or longer.

According to the claimed methods, long-term persistent vectors will include one in which the minichromosomal maintenance element consists essentially of the region of BPV mapping to positions 7590 to 7673; or wherein the minichromosomal maintenance element comprises (BPV E2 binding sites 6, 7 and 8) x, wherein x is 3 to 6 or wherein the minichromosomal maintenance element comprises at least 2 of the 3 E2 binding sites 6, 7 and 8.

The invention therefore also encompasses a recombinant vector for stable long-term persistence of erogenous DNA in a mammalian host cell, the vector comprising a minimal origin (MO) of replication of a papillomavirus, a minichromosomal maintenance element (MME) of a papillomavirus, and a gene encoding a gene product of interest, wherein the vector is defined hereinbelow (1-4).

1. The vector comprising papilloma virus sequences consisting essentially of (A) a minimal origin of replication of a papilloma virus, (B) a minichromosomal maintenance element of a papilloma virus consisting essentially of at least two of the three E2 binding sites 6, 7, and 8, wherein the region of the vector comprising the minimal origin of replication and minichromosomal maintenance element consists of a DNA sequence different from the natural papilloma virus sequence, and wherein the vector, when present in a mammalian host cell which expresses E1 and E2, persists in the cell for at least about 50 cell generations in dividing cells or for at least about 8 weeks in non-dividing cells under nonselective conditions without an appreciable loss of copy number.

2. The vector comprising papilloma virus sequences consisting essentially of (A) a minimal origin of replication of a papilloma virus, and (B) a minichromosomal maintenance element of a papilloma virus consisting essentially of multiple E2 binding sites, wherein the distance between the minimal origin of replication and the minichromosomal maintenance element is less than about 1.0 kb, wherein the vector, when present in a mammalian host cell which expresses El and E2, persists in the cell for at least about 50 cell generations in dividing cells or for at least about 8 weeks in non-dividing cells under nonselective conditions without an appreciable loss of copy number.

3. The vector comprising papilloma virus sequences consisting essentially of (A) a minimal origin of replication of a papilloma virus, (B) a minichromosomal maintenance element of a papilloma virus consisting essentially of the region of BPV mapping to about positions 7590-7673 wherein the vector, when present in a mammalian host cell which expresses El and E2, persists in the cell for at least about 50 cell generations in dividing cells or for at least about 8 weeks in nondividing cells under nonselective conditions without an appreciable loss of copy number.

4. The vector comprising papilloma virus sequences consisting essentially of (A) a minimal origin of replication of a papilloma virus, and (B) a minichromosomal maintenance element of a papilloma virus consisting essentially of (BPV E2 binding sites 6, 7, and 8)x wherein x is 3-6, wherein the vector, when present in a mammalian host cell which expresses E1 and E2, persists in the cell for at least about 50 cell generations in dividing cells or for at least about 8

weeks in nondividing cells under nonselective conditions without an appreciable loss of copy number.

As used herein, the term "consisting essentially of means that, with respect to papillomavirus sequences, those sequences which are both necessary and sufficient for long-term vector persistence without an appreciable loss of copy number.

In a preferred embodiment of the invention, a vector of the invention will comprise papillomavirus sequences as well as other sequences relating to expression of a gene of interest. The papillomavirus sequences in the vector will preferably consist essentially of a papillomavirus MME and MO, and thus will not contain papillomavirus sequences that are not required for long-term stable persistence in a eukaryotic host cell. The vectors thus advantageously do not contain papillomavirus sequences which are not both necessary and sufficient for long-term persistence in the episomal state. In addition, the vectors do not contain oncogenic sequences which are present in the papillomavirus genome.

In preferred embodiments, the minichromosomal maintenance element of a papillomavirus is from BPV; the minimal origin of replication of papillomavirus is from BPV; the papillomavirus El protein is from BPV; the papillomavirus E2 protein is from BPV.

Preferably, the vector further comprises a gene or genes encoding papillomavirus E2 and/or E1 proteins, and the E2 gene most preferably encodes a mutant form of E2 which is a point mutant that is replication competent but defect in transcriptional activation. As used herein, a "point mutant" may refer to either a single amino acid change, or several individual amino acid changes (2, 3, 4 etc.) which together confer the desired phenotype. A point mutant may be an amino acid substitution or a deletion or insertion.

One particularly useful form of a vector of the invention is a recombinant vector or vector system for stable persistence of erogenous DNA in a host cell, the vector comprising a minimal origin of replication of a papillomavirus, a minichromosomal maintenance element of a papillomavirus, and one or both of the papillomavirus E1 and E2 genes.

The invention also encompasses a mutant form of a papillomavirus E2 protein wherein the replication function of the protein is competent and the transcriptional activation function of the protein is defective, wherein the mutant form of E2 protein differs from the wild-type E2 in a nucleotide point mutation which translates into an amino acid substitution.

Preferred E2 point mutants are mutated in an alpha helical domain, for example in alpha helix 2 or 3, as defined herein.

Additional preferred E2 point mutants useful according to the invention are R37A, E74A, and D 122A and D143A/R172C.

A particularly striking feature of the invention is that the stable vectors of the invention are not restricted to the host cell specificity of papillomavirus. This release from the natural papillomavirus host cell type restriction has been achieved by removing key genetic elements of the papillomavirus genome from their native context; for example, expression of the papillomavirus genes encoding El and E2 proteins is not controlled by the promoters that are native to these genes, but rather the El and E2 genes are placed under the control of non-native, i.e., heterologous promoters, which are either functional in a broad range of mammalian cells or tissues or are cell- or tissue-specific.

It is preferred according to the invention that the expressible papillomavirus gene encoding El or E2 include a structural gene encoding El or E2 operatively associated with regulatory sequences for expression of the structural gene in a host cell. Such regulatory sequences will include a promoter and /or may optionally include an enhancer. The promoter is preferably a promoter that is non-native (i.e., heterologous) to the El or E2 structural gene. The promoter is may be functional in more than a single tissue type, i.e., one that is able to initiate transcription in a broad range of tissue types, and therefore unrestricted with respect to its tissue specificity. Alternatively, the promoter may be functionally restricted to a single tissue type, and therefore tissue-specific.

As used herein, tissue-specific and cell-type-restricted both refer to wherein a promoter is operable substantially in the same tissue-type or cell-type.

Preferred promoters comprise one of the thymidine kinase promoter and a strong promoter such as the SRalpha promoter. It is expected that a vector of the invention which includes tissue-specific regulatory elements operatively associated with the E1 and/or E2 genes will be capable of long-term persistence only in those cell types in which the regulatory elements are functional.

In its most useful form, a recombinant vector of the invention will include an expressible gene of interest.

A vector of the invention which contains an expressible gene of interest contains not only a structural gene encoding a protein or RNA of interest, but also is operatively associated with regulatory sequences for expression of the structural gene in a host cell. Such regulatory sequences may include not only a promoter, but also additional regulatory sequences such as an enhancer, splice sites, and poly-adenylation sequences. These regulatory elements that control

expression of the structural gene promoter may be regulatory elements that are native to the structural gene (i.e., the control sequences that are naturally associated with these genes in their native environment), or they may be non-native to the structural gene, and therefore heterologous regulatory elements. These elements, particularly the promoter, may be functional in more than a single tissue type or may be functionally restricted to a single tissue.

It is expected that a vector of the invention which includes a tissue-specific regulatory element that is operatively associated with a structural gene of interest will express that gene of interest only in those host cell types in which the regulatory elements are functional (i.e., specific).

It is preferred according to the invention that the host cell type-restricted expression (i.e., tissue-specificity) of the structural gene of interest be coordinated with the tissue-specificity of the regulatory elements operatively associated with the El and E2 genes. That is, one may envision that the tissue-specificity of El, E2, and structural gene of interest is the same. Alternatively, the tissue-specificity of E1 and E2 gene expression may be broader than the tissue-specificity of expression of the gene of interest, resulting in a broad host cell type range for long-term persistence of a vector of the invention, and a more limited host cell type range for expression of the gene of interest. Alternatively, the tissue-specificity of E1 and E2 gene expression may be quite limited (for example, to a single cell type), and the tissue-specificity of expression of the gene of interest broad or unlimited, resulting in a limited host cell type in which a vector of the invention can persist long-term, which in turn is the limiting factor in determining the type of host cell in which the gene of interest is expressed.

In another preferred embodiment of a vector of the invention, the vector also includes a bacterial host cell origin of replication and a gene encoding a selectable marker for preparation of vector DNA in a bacterial host cell.

The invention also features host cells containing the vectors herein described, such host cells being most preferably being eukaryotic, and of mammalian origin, such as of muscle, gut, or brain origin.

The invention also features a method of manufacture of a vector, which method includes culturing a host cell containing a vector described herein. It is particularly preferred that such manufacture occur in a lower eukaryotic cells, e.g., yeast or insect, or prokaryotic cells, e.g., bacterial cells such as E.coli or Salmonella. Therefor, the vector will further include an origin of replication of yeast, insect or bacterial origin, and one or more genes encoding a selectable marker, e.g., a gene encoding kanamycin resistance, for selection of cells containing the vector.

The invention also features a method of obtaining stable expression of a gene of interest in a cell, comprising providing a host cell as described above.

The invention also features methods of treating a disease stemming from a genetic defect, comprising administering a therapeutically effective or a prophylactic amount the vector of the invention to a patient afflicted with the disease.

The invention also includes use of a recombinant vector of the invention in the treatment of a disease.

The invention also encompasses a gene delivery system comprising a vector of the invention in combination with a gene delivery vehicle, which may be of viral or non-viral origin.

The invention also encompasses a method of producing a protein or RNA of interest in a host cell or a transgenic animal, comprising culturing a host cell under conditions which permit production of the protein of interest, or providing a transgenic animal which produces the protein, as described herein.

The invention also encompasses a mammalian model of disease, for screening of drugs to treat the disease or for testing of therapeutic or prophylactic regimes, the mammalian model comprising a transgenic animal whose cells contain a vector of the invention.

The invention also encompasses a transgenic animal containing an episomal vector as described herein, the vector encoding a protein of interest.

As used herein, a "transgenic animal" refers to an animal, preferably a mammal, which contains in some, but not necessarily all, of its cells an episomal vector, as described herein.

The invention also features kits for providing stable persistence of a vector in a host cell, the kit comprising a vector or a host cell as described herein and packaging materials therefore.

A kit of the invention may also include a mutant E2 protein as described herein or a gene encoding this protein, wherein the E2 mutant is thus provided for stable persistence of a vector in a host cell.

Uses and advantages of the invention are as follows.

The invention is useful in <u>in vivo</u> and <u>ex vivo</u> human gene therapy where correction of inherited or acquired genetic defects is desired. The invention also is useful in vaccination protocols where resistance or immunity to infectious pathogens, such as HIV, Hepatitis C Virus, Hepatitis B virus, Herpes virus, parasitic pathogens such as Tuberculosis and Leishmaniasis, and

protozoans such as ameobic dysentery, is desired, or the elimination or induced quiescence of aberrant cells, such as cancer cells, is considered beneficial.

Recombinant vectors of the invention are useful in that they permit persistent expression of a therapeutic gene in both dividing and non-dividing cells; for example, in differentiated cells, such as those in brain, and muscle.

Recombinant vectors of the invention are also useful for high level transient expression in cells where desired, such as for cancer therapy or in vivo vaccination.

Both in vivo and ex vivo gene therapy strategies are possible with this vector system, including stable, multicopy gene maintenance and expression, in haemopoietic and other stem cells, and in the committed and differentiated progeny of these cell types.

For human gene therapy, uses of the recombinant vectors of the invention are not limited in terms of delivery of the vector to a cell. That is, vectors of the invention may be delivered to a cell via non-viral or viral delivery systems. Delivery systems of non-viral origin include those which employ cationic liposomes, where vector size constraints do not limit the nature and number of plasmid vector components. Delivery systems of viral origin include viral particle-producing packaging cell lines as transfection recipients for the above E1/E2/M0/MME-containing plasmids into which viral packaging signals have been engineered, such as those of adenovirus, herpes viruses and papovaviruses.

Recombinant vectors of the invention also are useful in transgenesis, including production of transgenic animals via pronuclear injection, or embryonic stem cell transfection and embryo chimera generation.

Recombinant vectors of the invention also are useful for preparation of cell factories for stable, high level expression of proteins of therapeutic value in cultured mammalian cells.

Further features and advantages of the invention will become more fully apparent in the following description of the embodiments and drawings thereof and from the appended claims.

Description of the Drawings

Before describing the invention in detail, the drawings will be briefly described.

Figure 1A is a scheme of the experimental protocol.

Figure 1B is a short term replication assay for the plasmids in the CHO4.15 cells. Low molecular weight DNA was extracted from the CHO4.15 cells transfected with the plasmids containing minimal origin or 2.5 kb BglII fragment and analyzed by Southern blotting after

digestion with DpnI and linearizing enzyme XbaI (lanes 1, 2) and HindIII (lanes 3, 4). Lanes 1, 2 - episomally replicating minimal origin containing plasmid DNA extracted 36 and 60 hours after transfection and lanes 3,4 - episomal DNA extracted 36 and 60 hours after transfection with the plasmid containing 2.5 kb origin fragment.

Figure 2A stable replication of the BPV-1 origin containing plasmids in the CHO4.15 cell line. Representation of the BPV-1 fragment (BPV nucleotides 6945-1515) used in this experiment. The respective mutants in this fragment are depicted and are further described in Materials and Methods. The following genetic elements are indicated: NCOR-1 and 2 - Negative Control Of Replication 1 and 2; PMS-1 - Plasmid Maintenance Sequence; ORI - minimal origin of replication; E2RE1 and E2RE2 - E2 responsive enhancer 1 and 2; CE- constitutive enhancer; El, E6, E7 and E8 -respective ORF-s; P_{7175}, P_L, P_{89}, P_{890} - respective promoters; boxes indicate location of 14 E2 binding sites in this fragment.

Figure 2B is a southern blot analysis of stable cell lines for the presence of episomal plasmids. The marker lanes 1, 2, 3 - contain 100 pages of linearized vector pNeo5', pNeoBgl40 and pNeoBg1II, respectively. Low molecular weight DNA was extracted from CHO4.15 cells after transfection and G418 selection (see Materials and Methods for details). The plasmids used were the vector plasmid pNeo5' (lane 5), pNeoXhoI→HpaI with disrupted El protein binding site (lane 6), pNeoBgl40 (lane 7), pNeoΔNCORI with deleted Negative Control Region (lane 8), mutants Sma$^-$, 775 and 576 with disrupted 5'-part of El, E6 and E6/E7 ORFs respectively (lanes 9-11), the wild type pNeoBg1II (lane 12), and the minimal origin containing plasmid pNeoMO (lane 13).

Figure 3A shows stable extrachromosomal replication of the plasmids with deletions in the URR in CHO4.15 cells. Representation of BPV- I fragments with respective deletions.P_{7175}, P_L, P_{7940} - respective promoters in this fragment, E2REI and E2RE2 - E2 responsive enhancer, CE constitutive enhancer, boxes indicate E2 protein binding sites. End points of the respective deletions are given in Materials and Methods. Circle indicates the location of the minimal origin. Ability of respective deletion mutants to function in long term replication assay is indicated by (+) or (-).

Figure 3B. Low molecular weight DNA was extracted from the cells, transfected with respective plasmids and selected for G418, digested with the linearizing enzyme HindIII and analyzed by Southern blot (lanes 1-22). Lanes 23, 24 and 25 contain 100 pg of linearized pNeo5' vector, pNeoBgl40 and PNeoBglII marker DNA.

Figure 4A represents an analysis of state and copy-number of BPV-1 origin containing plasmids in CHO4.15 cells. Copy-number measurement of BPV plasmids stably replicating in CHO4.15 cells. Total DNA was extracted from the stable cell lines and subjected for linearization with HindIII. Lanes 1-3 represent analysis of 2 μg of total DNA from three independent cell-lines, a series of plasmid dilutions for copy-number reconstruction is included in lanes 4-6.

Figure 4B shows plasmid DNA, wherein a total of DNA 2 μg, cut with plasmid noncutter ApaI, from four independent cell-lines is analyzed (lanes 1-4). Respective marker of uncut plasmid DNA is shown in lanes 5 and 6.

Figure 5 shows restoration of stable replication of the plasmids by an MME consisting of oligomerized E2 binding sites. Low molecular weight DNA was extracted from the G418 resistant cells and linearized with HindIII (lanes 1-4) or with XbaI (lane 5). Lane 1 represents analysis of the DNA from the cells transfected with original D234/221 mutant with deleted MME. Insertion mutants with E2REI (BPV nucleotides 7611-7673) cloned into D234/221 adding back 18 and 9 E2 binding sites restored stable replication of the plasmid (lanes 2,3). Mutant with oligomerized 10 E2 binding sites cloned into D234/221 (lane 4) and mutant with deleted PMS1 with inserted 10 oligomerized E2 binding sites (lane 5) replicate in the long term assay. Lanes 6-8 contain 100 ng of linearized respective marker DNA.

Figure 6 shows the transformation efficiency (colonies per 100.000 cells) for G418 resistance of the plasmids pNeoBgl40 (Bgl40), pNeoXhoI→HpaI (HpaXho), pNeoMO (MO) and vector pNeo5'. 500 ng of the respective plasmid DNA was electroporated into the cells and 84 hours later cells were trypsinized, counted and plated. Complete medium containing 450 μg/ml of G418 was used for selection. Colonies were counted at the 10th day of selection. Presented values are average of three independent measurements. A. CHO4.15 cells, B. CHO212 cells and C. CHO49 cells.

Figure 7A is a schematic illustration of a plasmid in which E1 expression is under the control of the SR promoter.

Figure 7B is a schematic illustration of a plasmid in which E1 expression is under the control of the Thymidine kinase promoter.

Figure 7C is a schematic representation of the plasmid maps shown in Figs. 7A and 7B.

Figure 8 represents a comparison of the stable replication modes of BPV origin containing plasmid and chromosomal DNA in the CHO4.15 cells. BrdU labeling of the CHO4.15 cells carrying stably replicating pNeoBgl40 was done for a) and e) - 3.5, b) and f) - 9.5, c) and g) - 15.0 and d) and h) - 24 hours, respectively. Episomal - a), b), c), d) and total chromosomal DNA

- e), f), g), h) were prepared at respective time points and analyzed as described in Materials and Methods. CsCl gradients were aliqoted, denatured, renatured and loaded onto the nylon filters by slot-blotter and hybridized with radioactive BPV-1 origin probe for episomal DNA and with radioactive total CHO DNA for genomic DNA gradients. Intensity of hybridization was quantitated by use of the Phosphoimager.

Figure 9 shows the copy number of BPV-1 origin-bearing plasmids is similar in G1 and G2 phases of the cell cycle. Southern blot analysis of plasmid copy number in G1 and G2 phases of the cell cycle. Derivatives of CHO4.15 cell line with stably, extra chromosomally replicating BPV-1 origin-containing plasmids were arrested in G1/S with mimosine or hydroxyurea and in G2 with hydroxyurea, followed by Hoecsht 33342 treatment. Arrest was verified with FACS analysis, total DNA was extracted and an equal amount of DNA was loaded onto each lane. Analysis of four different established cell lines is shown on the figure with 3 parallels for each cell cycle arrest.

Figure 10 shows transient replication of pSR alpha and pTk vectors (Figure 7) in CHO (lane 1-4) and C333A (lanes 7-10) cell lines. 2 micrograms of respective plasmid was transfected into the cells by electroporation, time points were taken 36 and 48 hours post-transfection and low-molecular weight DNA was extracted, digested with DpnI and HindIII and analyzed by Southern blotting. Lanes 1-2 and 7-8 represent transient replication of SR alpha vector in CHO and C33A cells respectively. Over-replication is clearly visible in case of both cell lines. Lanes 3-4 and 9-10 represent transient replication of TK vectors in CHO and C33A cells respectively. Some over-replication is detectable in C33A cells, whereas replication of TK vector in CHO cells is weak, but without smear. The latter is characteristic for onion-skin type over-replication. Lanes 5-6 and 11 represent respective molecular-weight markers.

Figure 11 shows beta-galactosidase activity in CHO cells transfected with equimolar amounts of vector plasmids. In plasmids pON260 beta-galactosidase gene is expressed from immediate early CMV promoter. In other vector constructs beta-galactosidase is expressed from RSV LTR.

Figure 12 shows beta-galactosidase assay for the cells transfected with the pSR alpha and pTK constructs after 56 hours. In the case of TK constructs formation of lacZ- positive blue colonies could be detected, however in the case of SRalpha constructs only single, strongly stained, blue cells could be detected in the culture indicating that plasmids are not inherited to each of the daughter cells during cell division.

Figure 13A shows a number of colonies per 10000 cells transfected with equal molar amounts of vectors selected for G418.

Figure 13B is an analysis of the episomal DNA in the cells transfected and selected for G418 with the TK-E1 constructs.

Figure 14A is a schematic representation of the designed E2 point mutations and chimeric E2 proteins.

Figure 14B shows an immunoblots of the E2 proteins.

Figure 15A shows the transient replication properties of the mutated E2 proteins in CHO cells.

Figure 15B shows the structure of the reporter plasmids used. The numbers indicate nucleotide positions in the BPV URR sequence. pUCAlu was used for transient replication studies. pPCAT and pSV3BS9CAT are the CAT reporter plasmids used in transcriptional activation assays.

Figure 16 represents a comparison of transactivation and DNA binding abilities of E2 protein mutants.

Figure 17 shows results of a transient transcription assay for the E2 protein mutants. A. Mutations with nearly wild-type properties in transient transcription assay. B. Mutations, which transcriptional activity has decreased to 50% of that of the wild-type protein. C. Inactive mutations for transcription. D. Transcriptional properties of chimeric proteins p53:E2 and VPI6:E2.

Figure 18 shows results of staining for beta-galactosidase activity in brain striatum sections, where panels A-F represent plasmid DNA dissolved in cerebrospinal fluid (A-D) or PBS (E, F).

Figure 19 is a bar graph showing results of β-galactosidase reporter gene expression in mice after injection of a vector of the invention containing a β-galactosidase gene.

Figure 20 provides the nucleotide and amino acid sequence of the BPV1 E2 sequence (SEQ ID NO: 1). The positions which were mutated to produce E2 point mutants are designated as * and are referred to in the text by amino acid residue. The E2 protein sequence begins at the MET indicated as 1.

Figure 21 provides the nucleotide sequence of the BPV upstream regulatory region (SEQ ID NO:2). The MME sequence is located between positions 7475(Cla 1 site) and the Hpa 1 site (7947).

Description

The invention is based on the recognition that DNA replication in papillomavirus from the minimal origin (MO) *per se* is not sufficient for long-term persistence, but that in addition another viral sequence is required. This sequence, termed herein a minichromosomal maintenance element (MME) comprises a binding site for proteins which are essential for papillomavirus replication. Although the MME sequence may include binding sites for replication proteins that are of viral or human origin, for example, in BPV, the sequence appears to be dependent on viral proteins E1 and E2, and is specifically bound by the viral E2 protein, when the sequence is linked to the minimal origin sequence.

The invention thus is based on the discovery of a cis-acting element, referred to herein as a minichromosomal maintenance element (MME), which confers long-term stability to a transiently replicating eukaryotic episomal plasmid. An MME is distinct from a minimal origin of replication (MO), and is required in addition to an MO for long-term plasmid persistence in a host cell.

RECOMBINANT VECTORS OF THE INVENTION

The invention encompasses a genetic construct comprising a single plasmid containing the MO and MME sequences (MO/MME vectors) and a cloning site for insertion of a gene of interest. The invention also encompasses a genetic construct comprising a single vector containing genes encoding E1 and E2 plus MO and MME sequences (E1/E2/M0/MME vectors) and a cloning site for insertion of a gene of interest. In its most useful aspect, the invention features the above described plasmids wherein a gene of interest also is contained in the plasmid.

Optionally, vectors of the invention may include multiple cloning site cassettes and selectable markers conferring drug resistance to mammalian and bacterial cells, and reporter genes such as lacZ (Figure 7). These vectors can be used as stable expression vectors in a wide range of both dividing and non-dividing (post-mitotic) cell types.

An important property of a plasmid containing these determinants is that replication of this episomal plasmid is not subject to regulation by the cellular controls which regulate host genome replication; that is, replication occurs independently and is under EI /E2 control in the S-phase of the cell cycle. (Figure 8). The initiation of EI/E2-dependent replication of MO/MME- containing plasmids is random in the cell cycle but over- replication does not occur; stable copy number is maintained in both G1 and G2. (Figure 9).

Vectors of the invention are safe to use in human cells and impart no known oncogenic properties to recipient cells. All papilloma-encoded oncogenic sequences have been deleted. (Figs. 2 and 3).

Definitions

As used herein, "papillomavirus" refers to a member of the papilloma family of viruses, including but not limited to bovine papillomavirus (BPV) and human papillomavirus (HPV).

"Minimal origin of replication" (MO) refers to a minimal cis-sequence within a papillomavirus that is necessary for initiation of DNA synthesis. The MO of BPV-1 is located at the 3' end of the upstream regulatory region within a 60 μ fork, corresponding to a 52 base pair DNA fragment (7928-7947/1-25) including an AT-rich region, a consensus sequence to which all papilloma viral E2 proteins bind, and an E1 protein binding site spanning nucleotide 1. The MO of HPV is located in the URR fragment (for example, in HPV11 at nt 7072-793 3/1-99) (Chiang *et al.* PNAS 1992).

In a transient replication assay, the efficiencies of replication of plasmids bearing a Minimal Origin (MO) and a full size Origin of Replication are equal. Only two viral proteins, E1 and E2, are required for stable replication of the full size Origin. An observation which led in part to the discovery which forms the basis of the invention is that the minimal origin of replication (MO) is absolutely essential, but it is not sufficient to stably maintain the plasmids in an episomal state; additional elements in the Upstream Regulatory Region are not only required, but are sufficient, for stable persistence of the plasmids (Figure 2).

"E1" refers to the protein encoded by nt 849-2663 of BPV subtype 1; or to nt 832-2779 of HPV of subtype 11, or to equivalent E1 proteins of other papillomaviruses, or to functional fragments or mutants of a papillomavirus E1 protein, i.e., fragments or mutants of E1 which possess the replicating properties of El.

"E2" refers to the protein encoded by nt 2594-3837 of BPV subtype 1; or to nt 2723-3823 of HPV subtype 11, or to equivalent E2 proteins of other papillomaviruses, or to functional fragments or mutants of a papillomavirus E2 protein, i.e., fragments or mutants of E2 which possess the replicating properties of E2. Numerous E2 mutants are described herein which are defective in the E2 transcriptional activating activity and competent in the replicating ability.

"Minichromosomal maintenance element" (MME) refers to a region of the papilloma viral genome to which viral or human proteins essential for papilloma viral replication bind, which

region is essential for stable episomal maintenance of the papilloma viral MO in a host cell. Preferably, the MME is a sequence containing multiple binding sites for the transcriptional activator E2, although the MME also may be a sequence containing host cell factor binding sites. An MME is most likely to be located in the upstream regulatory region of the viral genome. The MME in BPV is herein defined as the region of BPV located within the upstream regulatory region which includes a minimum of about six sequential E2 binding sites, and which gives optimum stable persistence with about ten sequential E2 binding sites, and which may include as many as about 20-30 or as many as about 50 sequential E2 binding sites. The sequential binding sites which constitute the MME need not be identical in sequence, but must be able to bind E2. In addition, between each sequential binding site in the MME, there may be spacer nucleotides, for example, 6 nucleotides, sufficient to insert a restriction enzyme site. The spacer nucleotides may be absent from the MME or may extend to a length of 10, 20 or even 50 nucleotides, so long as the binding of E2 to each separate binding site is not disrupted by the presence of the spacer.

The Minichromosome Maintenance Element (MME) of BPV comprises multiple, binding sites for the E2 protein (Figure 3). 10-20 tandem repeats of E2 binding sites impart greater stability and higher plasmid copy number (approx. 30 copies per cell, Figure 4) in cells expressing the BPV (or HPV) El and E2 proteins. (Figure 5). It is believed that MME/M0-bearing plasmids function in a wide range of eukaryotic cells including rodent, monkey and human cells, and in almost any tissue type.

The MME confers (E1+E2)-dependent stable replication upon plasmid transfection into a host cell line expressing both E1 and E2. It is observed that neither E1 nor E2 alone is sufficient to permit MME-mediated plasmid stability (Figure 6). E1 and E2 can be provided to the plasmid either in cis or in trans (i.e. from integrated E1 and E2 expression vectors or from the same episomal plasmid).

"E2 binding site" refers to the minimum sequence of papillomavirus double-stranded DNA to which the E2 protein binds. This binding site is in most papillomaviruses located in the upstream regulatory region in BPV and HPV. In BPV, the E2 binding site is a palindromic 12 nucleotide sequence (ACCN6GGT, where N is any nucleotide) which is repeated approximately 10 times within the URR. The affinities of the 10 E2 binding sites for E2 varies among the binding sites in the BPV URR, with site 9 (ACCGN4CGGT, where N is any nucleotide) being the strongest E2 binding site.

A "host cell" which is stably transformed according to the invention may be any prokaryotic or eukaryotic cell, an is preferably a mammalian cell, and most preferably a human cell. The cell may be derived from any tissue, for example, muscle, nerve tissue, etc.

When the E1 and/or E2 genes are located in cis with respect to the MO and MME, this refers to a genetic context in which one or both genes are located on the same episomal element or the same vector as the MO or MME. In contrast, when the E1 and/or E2 genes are located in trans with respect to the MO and MME, this refers to a genetic context in which the E1 and/or E2 genes are not located on the same episomal element as the papilloma MO or MME, such as a context in which the El and/or E2 genes are integrated into the host cell chromosome or the El and E2 genes are carried on a separate (non-contiguous) genetic element (vector or episome).

"Stable maintenance" or "long-term persistence" refers to two characteristics of vectors of the invention. First, it refers to the ability of a vector according to the invention to persist or be maintained in undividing cells or in progeny cells of dividing cells in the absence of continuous selection over the long-term. As used herein, "long-term" refers to a period of time that is longer than at least about 5 weeks, for example longer than 8 weeks (where a given cell doubling time is about 16 hours). Of course, for a longer or shorter cell doubling time, the definition of "long-term" changes accordingly. Second, it refers to the ability of a vector according to the invention to persist without an appreciable loss of copy number from one cell division to the next. In determining whether a given vector is capable of long-term persistence, the recombinant vector may be introduced into the host cell under conditions in which the vector is selected for, when the selection conditions are thereafter removed, the copy number of the recombinant vector nevertheless remains constant and reliable thereafter; for example, in non-dividing cells, a vector of the invention persists for a period that is longer than at least 5 weeks, for example, 6 weeks, 8 weeks, 12 weeks or longer, for a host cell which doubles in about 16 hours. A vector of the invention is capable of persisting, in dividing cells, over about at least 50 generations, 60 generations, 80 cell generations, or 120 cell generations or longer under non-selective conditions without an appreciable loss of copy number. We have found that loss of copy number over 80 generations of cell doublings is less than about 10% (over 80 generations). Therefore, loss of copy number from one cell generation to the next is less than about 0.125% or about 0.1%. Therefore, the vectors of the invention are not subject to an appreciable loss of copy number from one cell generation to the next. In contrast, "short-term persistence" of a plasmid in a host cell refers to the inability of a plasmid to persist in a host cell long-term, as defined herein, without an appreciable loss of plasmid copy number, as defined herein.

A "gene of interest" refers to a gene encoding a gene product of interest such as a protein of interest or an RNA of interest. A "protein of interest" refers to any therapeutic, prophylactic or marker protein useful according to the invention. In addition to any therapeutic protein selected for a treatment using a vector of the invention, a therapeutic protein may include a cell or viral surface antigen to which an immune response may be elicited when used in a vaccine.

"Heterologous" or "erogenous" gene refers to a coding sequence that is introduced into a host cell on a vector of the invention. The coding sequence may be identical to a sequence contained within the host cell, or it is most likely not identical to a host cell sequence.

"Heterologous promoter" refers to a promoter that is not the natural (or homologous) promoter that initiates transcription from the gene with which it is associated, whether that gene be the gene encoding a protein of interest or the genes encoding the El and E2 proteins. A "strong" promoter, with respect to E1 and E2 expression, refers to a promoter which supports overexpression of the E1 and/or E2 gene to an extent that the vector is maintained in sufficiently high copy number so as to make the host cells unhealthy. An example of a strong promoter is the SR-alpha promoter; other strong promoters will provide about the same level of expression of a reporter gene in an assay for quantitating gene expression and thus promoter strength, e.g., a betagalactosidase assay.

As explained hereinabove, a vector useful in the invention will contain papillomavirus MO and MME sequences, as defined herein, and a cloning site for insertion of a gene of interest, and will require the presence of the papillomavirus El and E2 proteins for long-term persistence in a host cell. The E1 and E2 proteins are effective in trans in the cell and therefore the genes encoding these proteins may be present in trans with respect to the vector, or they may be present in cis, i.e., within the vector DNA. In addition to the above-described sequences, a vector useful in the invention may contain a heterologous gene encoding a protein of interest and also may contain sequences for regulation of that gene.

Described below are experiments in which the BPV MME is localized and characterized. This experimental strategy also is useful for localizing and characterizing the HPV MME. Following the description of the BPV MME characterization is a description of the components of vectors of the invention; i.e., heterologous genes of interest in vectors of the invention, heterologous regulatory sequences, e.g., promoters, which may be used to direct El and E2 gene expression and to direct heterologous gene expression, E2 mutants useful according to the invention, host cells useful in the invention, and delivery vehicles useful for delivering vectors of

the invention to host cells. The Examples provided hereinbelow describe specific embodiments of the invention and are meant to illustrate and not to limit the applicability of the invention.

Localization of BPV MME Sequence

We have constructed a cell line CHO4.15 expressing constitutively the viral proteins El and E2, that are required for initiation of viral DNA replication. It has previously been demonstrated that the minimal origin of replication and the viral El and E2 proteins are sufficient for plasmid replication. It is demonstrated herein that the E1 and E2 viral proteins are not only necessary, but are sufficient when coupled with the MME for long-term episomal persistence.

Using the cell line CHO4.15 it is shown herein that the BPV-1 minimal origin of replication (MO) is absolutely necessary, but is not sufficient for stable extrachromosomal replication of viral plasmids. By deletion and insertion analysis, an additional element (Minichromosome Maintenance Element - MME) in the Upstream Regulatory Region of BPV-1 has been identified which assures stable replication of the MO containing plasmids. This element is composed of multiple binding sites for the transcriptional activator E2. MME appears to function in the absence of replication but requires El and E2 proteins for activity. In contrast to EBV or EBV oriP-containing plasmids, for example, stably maintained BPV-1 plasmids are not subject to once-per-cell cycle replication as determined by density labeling experiments. These results indicate that papillomavirus episomal replicators replicate independently of the chromosomal DNA of their hosts.

Construction of cell lines expressing El and E2 proteins

The E1 and E2 proteins of BPV-1 are necessary for initiation of DNA replication from the viral origin of replication. Expression of these two proteins from heterologous expression vectors allows replication of a minimal origin in transient replication assays (Ustav and Stenlund, 1991; Ustav *et al.*, 1991). However, due to the lack of persistence of the transfected expression vectors, replication can not be monitored for more than few days after transfection.

To determine whether additional *trans*-acting factors or *cis*-acting elements are required for long term persistence of the viral DNA, continuous expression of these two factors has to be assured. We therefore constructed several cell lines constitutively expressing the El and E2 proteins. Expression of these proteins was directed from integrated constructs for El protein from

CMV promoter (cell line CHO212) and for E2 protein from HAP 70 promoter (cell line CHO49). In the cell line CHO4.15 which expresses both El and E2, the El protein was expressed from SRα-promoter and the E2 protein from HSP 70 promoter. Selection of the respective cell lines and amplification of the expression units of interest was achieved by utilizing the glutamine synthetase minigene from the PSVLGS.1 plasmid according to the protocol described earlier (Bebbington and Hentschel, 1987). Expression of El and E2 was identified by immunoprecipitation using specific rabbit polyclonal sera (data not shown) and by in vivo replication assays. The three cell lines and the parental CHO cells were transfected with the BPV-1 origin containing plasmid pUC/Alu in combination with El and E2 expression vectors. The cell line CHO4.15 which expresses both El and E2, supports replication of the origin plasmids in the absence of erogenous E1 and E2. The E2 expressing cell line, CHO49, supports replication in the presence of an E1 expression vector, but fails to do so without erogenous El. The El expressing cell line, CHO212, supports replication only in the presence of an E2 expression vector. In the parental CHO cell line, co-expression of both E1 and E2 is required for replication. No replication of pUC/Alu can be detected in the absence of E1 and E2.

Described below are experiments which demonstrate the trans and cis-acting elements which are not only necessary but are sufficient for long-term episomal persistence. The E1 and E2 proteins are both necessary and sufficient for stable replication of the BPV- I origin containing plasmids. In addition, the BPV- I URR contains sequences which are not only required, but are sufficient for long-term replication of the papilloma-derived episome.

This experimental strategy and procedure also may be used to determine the exact location in HPV of the MME, simply by substituting HPV for BPV, and testing regions of the HPV genome. Such testing would begin with the URR of HPV, which region includes binding sites for E2 and for cellular factors involved in replication.

The experimental protocol used for defining the BPV MME was as follows.

To determine what viral *trans*-acting factors and *cis*-sequences were required for long term replication of BPV-1, we used the CHO4.15 cell line which constitutively expresses both the viral E1 and E2 proteins. Different fragments of BPV-1 were cloned into the vector pNeo5' (Lusky and Botchan, 1984). This plasmid provides amino glycoside 3'-phosphotransferase as a marker for selection of the cells in the presence of geneticine (G418). We used a 2.5 kb BglII fragment from the BPV-1 genome (nucleotides 6946-1515) as a starting fragment. This fragment contains the URR including the E2 dependent transcriptional enhancer, the minimal origin of replication and part of the early ORF's (construct 12, Figure 2A). This plasmid, in parallel with a

minimal origin fragment in the same backbone (construct 13, Figure 2A), were transfected into the CHO4.15 cell line by electroporation and processed according to the scheme in Figure 1A.

In Fig 1A, both transient and long-term plasmid persistence was determined. A portion of the transfected cells were used after plating for analysis of transient replication. The remaining portion of the cells were selected in the presence of G418 for three weeks, colonies were then pooled or picked and grown under nonselective conditions for two additional weeks, to give a total of five weeks, at which time low molecular weight DNA was harvested and analyzed for the presence of replicated plasmid. The ability of the origin-containing plasmids to replicate extra chromosomally in transient and long term replication assays was examined by Southern analysis of the episomal DNA (see Materials and Methods for details). The two plasmids containing the 2.5 kb BglII fragment and the minimal origin respectively, replicated to comparable levels in the transient replication assay (Figure 1B). After selection in the long term replication assay, however, the result was very different. While the plasmid containing the 2.5 kb BglII fragment could be readily detected in episomal form, the minimal origin containing plasmid could not be recovered (compare lanes 12 and 13, Figure 2B). These results showed that sequences present in the larger plasmid, but absent in the plasmid containing only the minimal origin were required for long term replication, and that an activity in addition to replication was required for stable persistence of a plasmid in the CHO4.15 cells. These results also indicated that it was possible to maintain BPV origin containing plasmids for an extended period of time (e.g., five weeks) without the regulatory circuit that the viral genome can provide.

To determine what sequences within the BglII fragment were responsible for maintenance in the long term assay, we generated mutations within that fragment and assayed these plasmids for maintenance. Initially, we generated mutations in the sequences suggested previously to have effects on replication. The BPV-1 BglII fragment contains coding sequences for three potential proteins, E6, E7 and the N-terminal part of the E1 protein. Mutations which interrupted E1 ORF, E6 ORF and E6/7 ORF (Lusky and Botchan, 1985; Schiller *et al.*, 1984; Berg *et al.*, 1986) - construct 9 (pNeo Sma$^-$), construct 10 (pNeo775), construct 11 (pNeo576), respectively and in addition a deletion removing all coding sequences - construct 7 (pNeoBgl40), were introduced into the pNeo5' (Figure 2A). None of these mutations had a detectable effect on maintenance (compare lane 12 with lanes 7, 9, 10 and 11 Figure 2B) indicating that the coding sequences contained within the BglII fragment were dispensable. Consequently the E1 and E2 are the only viral gene products required for maintenance.

It has been suggested previously that BPV-1 URR contains two partially overlapping cis-regulatory control elements for stable replication, termed Plasmid Maintenance Sequence (PMS-1) (Lusky and Botchan, 1984) and Negative Control of Replication (NCOR-1) (Roberts and Weintraub 1986). In order to demonstrate that these two sequences were not necessary or sufficient for long-term plasmid persistence, each sequence was deleted by removing the sequence between the HindIII and MluI sites (nt. 6959-7351, construct 8 - pNeoΔNCOR, Figure 2A). This deletion had no deleterious effect on long term replication of the plasmid (lane 8, Figure 2B), demonstrating that these putative elements were not required. Finally, as a negative control, an XhoI linker insertion mutant overlapping with HpaI site was generated (construct 6, Figure 2A). This mutation generated an origin defective for replication in the transient replication assay, and it is also defective for long term replication (lane 6, Figure 2B). We concluded from these results that cis-elements required for stable replication of BPV-1 are located within the URR and are unrelated to the previously proposed elements PMS-1 and NCOR-1. We have named this cis-element in the BPV-1 URR Minichromosome Maintenance Element (MME).

MINICHROMOSOMAL MAINTENANCE ELEMENTS OF THE INVENTION

A minichromosome maintenance element is localized and defined as follows.

The Minichromosome Maintenance Element is composed of redundant sequences.

To define the sequences required for long term replication we generated a series of deletion mutants within the URR (Figure 3A). These deletions were made in the context of the plasmid pNeoBgl40 (BPV-1 sequence from 6946 to 63), and the deletion mutants were tested in the long term replication assay. The first series of constructs (1 -6, Figure 3A) had a fixed 5'-end (nucleotide 7187) and progressive deletions were made at the 3'-end of the URR (nucleotides 7892, 7834, 7771, 7475, 7389, respectively). The first four of these deletions (constructs 1-4, Figure 3A) were defective for stable replication (Figure 3B), but the plasmids with less extensive deletions (plasmids 5 and 6, Figure 3A) were maintained at the wild type level (lane 5 and 6, Figure 3B). Another set of deletions (constructs 7-11, Figure 3A) had a fixed 3'-end (nucleotide 7890) and progressive deletions from the 5'- end (7476, 7611, 7673, 7771, 7834, respectively). Two mutants of this series were unable to replicate stably (deletions 7 and 8, Figure 3A and 3B), but plasmids with less extensive deletions replicated efficiently (lanes 9-11, Figure 3A and 3B).

The results from the unidirectional deletions showed that a sequence in the vicinity of nucleotide 7600 was required for long term replication (compare constructs 4 and 5, and 8 and 9). To map this sequence more precisely we generated a third set of deletions. These deletions were constructed as scanning deletions (constructs, lanes 11 - 21, Figure 3A, 3B). Surprisingly, none of the introduced mutations resulted in loss of MME activity demonstrating that no single unique sequence within the URR was required, but that maybe some redundant sequence element was responsible.

The MME is composed of Binding sites for the E2 transcription activator.

To address directly if the sequences in the vicinity of nucleotides 7600 were responsible for the MME activity we generated a fragment between nucleotides 7590 and 7673, and inserted this fragment into the deletion mutant D221/234 (construct 1 in Figure 3A) to determine if replication in the long term replication assay could be restored (this fragment corresponds to the sequence between the deletion end-points D 134 and D11). This fragment inserted in three and six copies restored MME activity in the long term replication assay (Figure 5, compare lanes 1 with 2,3). A known constituent of this fragment are three high affinity E2 binding sites. A possibility that occurred to us was that the MME activity was contributed by E2 binding sites. This would be consistent with the apparent redundancy, since the URR contains 10 binding sites for E2. To determine if E2 binding sites were involved in MME activity we oligomerized a high affinity E2 binding site 9 of the BPV-1 URR (5'-ACCGTTGCCGGT-3') with six nucleotide spacing (Li *et al.*, 1989) and inserted these oligomers (10 copies) into D221/234 deletion mutant. This insertion restored the MME activity (lane 4, Figure 5). To rule out involvement of other BPV sequences we added 10 oligomerized E2 binding sites to the minimal origin of replication. Those constructs replicated with similar efficiency as plasmids with wild type BPV-1 sequences in the stable assay (lane 5, Figure 5). However, plasmids with less than six additional oligomerized E2 binding sites failed to replicate in the long term replication assay (data not shown). These results strongly suggest that binding sites for the E2 protein can be responsible for providing MME activity to the BPV-1 origin.

The MME enhances the frequency of formation of G418-resistant colonies without replication.

It has previously been observed that for EBV, multimerized EBNA-1 binding sites (Family of Repeats - FR) in an EBNA-1 dependent fashion are required for stable replication of oriP containing plasmids (Krysan *et al.*, 1989; Kirchmaier and Sugden, 1995; Middleton and Sugden, 1994). This activity can be measured by increased transformation frequency of the plasmids carrying FR, and is thought to be caused by enhanced nuclear retention of plasmids containing FR. To determine if a similar activity could be determined for MME we measured the transformation frequency of four different plasmids. First, pNeo5' carries the selectable neomycin resistance marker, but lacks BPV sequences and consequently is defective for replication in both the short term and long term replication assays. Second, the minimal origin plasmid, in addition carries the BPV minimal origin and is replication competent in the short term replication assay but not in the long term assay. The third plasmid pNeoHpaI→XhoI carries the whole Bgl40 fragment, and is thus nominally capable of maintenance, but because of the linker insertion in the E1 binding site the plasmid is defective for replication. The fourth plasmid pNeoBgl40 is replication competent in both the short and long term replication assays. We used CHO4.15 cells to measure transformation frequency of these different plasmids (Figure 6). The vector with the selectable marker only and the plasmid containing the minimal origin transformed CHO4.15 cells with similar frequency, 14 and 16 colonies per 10^5 cells, respectively. In the parallel experiment, pNeoHpaI→XhoI which is replication defective with a mutant E1 binding site, but carrying the sequences required for MME activity, transformed CHO4.15 cells 4 to 5 times more efficiently than vector alone or a plasmid with minimal origin of replication (68.6 colonies per 10^5 cells) (Figure 6A). The plasmids with the complete origin transformed cells with approximately 100 times higher efficiency than the vector containing only the minimal origin of replication (approximately 1600 colonies per 10^5 cells). These results indicated that MME activity could be measured in a stable transformation assay even in the absence of replication. When the same experiments were also performed in CHO212 (E1 cell line) and CHO49 (E2 cell line), all plasmids transformed with approximately the same efficiency in these two cell lines (Figures 6B and 6C). We conclude that enhanced transformation activity requires both E1 and E2 proteins.

We measured the possible effect of an MME consisting of oligomerized E2 binding sites on plasmid retention in the short term assay, as it has been done with the EBV oriP containing

plasmids. However, attempts to reproduce a direct effect on nuclear retention in transient assay failed to show a significant effect (data not shown).

BPV origin plasmids replicate approximately at 15 copies per haploid genome.

One of the factors that is expected to influence the stable persistence of a plasmid is the copynumber. We therefore performed experiments to estimate the average number of episomal copies per haploid genome in different established cell lines. After digestion with the single-cut restriction endonuclease (Hind III) total DNA from three independent cell lines - pNeoBgl 40, pNeo41/Cla and pNeoSca/234 was loaded in equal amounts onto the gel and was analyzed by Southern blotting using radioactively labeled BPV-1 origin and neo probe. All three cell lines contained approximately the same number of episomal plasmids - 15 copies per haploid genome (Figure 4A). Even though no specific effort was made to determine the number of integrated copies, digestion with a non-cutter enzymes did not change the appearance of the three forms and oligomers of the plasmid (Figure 4B). Consistent with previous reports, the majority of the plasmids were present in the oligomeric form. We conclude from these results that the plasmids are mostly episomal in the CHO4.15 cell line under the conditions used.

Mode of replication

One explanation for the apparent high stability of BPV-1 plasmids in the cells could be that the plasmids are subject to the cellular once per cell cycle replication control. To determine if this was the case we performed density labeling experiments using the cell line CHO4.15 containing the replicating plasmid pNeoBgl40. The experiments were performed by continuous labeling of the cells with BrdU for 3.5, 9.5, 15 or 24 hours. Low molecular weight DNA and total chromosomal DNA were extracted after each time point and analyzed by CsCl gradient centrifugation, followed by slot blotting, and hybridization with plasmid probe or genomic DNA probe to identify the peaks in the gradient. The density gradient profiles are shown in Figure 8. The data is summarized in the table as fractions of Bgl40 DNA and CHO chromosomal DNA that had incorporated no BrdU (light-light- LL), BrdU incorporated into one strand (heavy-light - HL), or into both strands (heavy-heavy - HH) in the CHO4.15 cells stably transformed by this plasmid (Figure 8). After labeling with BrdU for 3.5 hours (panels a) and e)) the episomal BPV-1 origin containing plasmids were divided between three forms of DNA: 5% heavy-heavy, 19%

heavy-light and 76% light-light, while chromosomal DNA is distributed between two forms 27% heavy-light and 73% light-light. After labeling for 9.5 hours, the plasmid has accumulated considerable amount (21%) of heavy-heavy DNA, while chromosomal DNA shows no detectable signal in the heavy-heavy area. After labeling for 15 hours, distribution of the episomal DNA is 34% heavy-heavy, 38% heavy-light, 28% light-light. At the same time chromosomal DNA showed still very little, if any, heavy-heavy DNA. After labeling for 24 hours, episomal DNA was preferentially in the heavy-heavy fraction of the DNA (66%), 24% heavy-light and 10% light-light, while chromosomal DNA showed considerable amount of heavy-heavy DNA (24%), but with most of the DNA still in the once replicated DNA fraction. These results are consistent with a doubling time of approximately 16 hours for the pNeoBgl40 containing cell lines. The considerable percentage of the unreplicated chromosomal DNA after 24 hours is likely to be due to growth arrest of a fraction of the cells by the conditions used for BrdU labeling. It appears clear from these results that the stably maintained pNeoBgl40 plasmid does not replicate once per cell cycle and stable persistence of the BPV-1 plasmids is not a function of once per cellcycle replication control.

Mechanism of Action

Three mechanisms of action can be envisioned for the MME. First, the MME could affect the efficiency of initiation of replication. Although no difference in replication initiation can be detected during the time-course of the transient replication assay (Ustav *et al.*, 1991), it is conceivable that a gradual accumulation of methylated residues at the origin of replication or some other form of modification (including nucleosome occlusion) could prevent initiation of replication and results in gradual loss of replication activity. It is possible that MME can affect these processes and prevent inactivation of the origin. Alternatively, the minimal origin containing plasmids are competent for over replication during the S-phase of the cell cycle, which could be toxic to cells. MME could prevent this process, in analogy to the function of iterons for certain bacterial plasmids (for review Nordström, 1991). Third, MME could influence the partitioning process thereby affecting the loss rate of plasmids during cell division.

It is interesting to note that the ability of the minimal origin containing plasmids to replicate appears to have no detectable effect on transformation frequency. One might have expected that an increase in the quantity of plasmid DNA in the cells as a result of replication would lead to a higher frequency of integration. However, this appears not to be the case,

possibly because these minichromosomes are poor substrates for the required recombination events or are lost with very high frequency at cell division. The very large increase in transformation frequency of the plasmids with both MO and MME compared to integrating marker presumably reflects the fact that the two functions together can bypass the requirement for integration possibly by providing an efficient segregation/partitioning function in addition to replication.

Materials and Methods

The following methods are routinely used in the invention. These methods are stated in terms of the detailed experiments performed herein. However, each method may be generalized by one of skill in the art for use in carrying out the invention in its broadest sense, as described below and claimed.

Plasmid construction. (i) Expression vectors. The E1 and E2 protein expression vectors pHSE2 (Szymanski and Stenlund, 1991), pCGE2 and pCGEag (Ustav and Stenlund, 1991) have been described earlier. The E1 expression vector pE1 - 1x5 contains the BPV-1 E1 ORF with XhoI linkers (within nucleotides 619 to 2757) and carries a point mutation at the splice donor site at nucleotide 1235. This fragment was cloned into the XhoI site downstream of the SRα - promoter in the plasmid pBJ5GS (kind gift from Dr. L.Berg).

(ii) Origin plasmids. All origin fragments of the BPV-1 genome were cloned in sense orientation into the BamHI site of the pNeo5'(Lusky and Botchan, 1984). pNeoBg1II contains a Bg1II fragment from BPV-1 (nt. 6946 to 1515) cloned in the sense orientation relative to the transcription of the neo gene. pNeoXhoI→HpaI contains the same BglII fragment with an XhoI linker insertion into the HpaI site (Ustav *et al.*, 1991). pNeo 576, pNeo775 and pNeoSma⁻ plasmids contain the same BglII fragment with the mutations 576, 775 and Sma- which affect E6/7 ORF, E6 ORF and the 5'-part of the E1 ORF, respectively (Lusky and Botchan,1985; Schiller *et al.*, 1984; Berg *et al.*, 1986). pNeoΔNCOR has a deletion between HindIII (nt. 6958) and MluI (nt.7351). pNeoBgl40 contained a BPV-1 fragment from nucleotide 6946 to 63, which was amplified by PCR using respective primers and cloned in sense orientation into the BamHI site. pNeoMO contained minimal origin sequence (nucleotides 7914 to 27) cloned into the BamHI site. Linker deletion mutants of the BPV-1 genome (Szymanski and Stenlund, 1991) were used as templates for PCR. Primers - 5'-AAAAGCTTTCTTTGGACTTAGA-3' (BPV-1 nucleotides

6959-6979) and 5'-ATAGCCAGCTAACTATAGATCT-3' (BPV-1 nucleotides 45 to 63 flanked by BglII site) were used to amplify origin fragments. PCR products were cloned into the HindIII and BamHI site of the pNeoBgl40. Deletion mutants lacked following sequence: D221/234 - 7187/7892; D36/234 7187/7834; D121/234 - 7187/7771; D134/234 - 7187/7673; DC1a/234 - 7187/7475; DSca/234 7187/7389; D221/Cla - 7476/7892; D221/11 - 7611/7892; D221/134 - 7673/7892; D221/121 7771/7892; D221/36 - 7834/7892; D36/121 - 7771/7834; D121/134 - 7673/7771; D134/43 - 76227673; D43/11 - 7611/7622; D11/229 - 7597/7611; D229/Cla - 7476/7597; DC1a/41 - 7355/7476; D41/136 - 7344/7356; D136/Nar - 7273/7344; DNar/64 - 7214/7273 and junctions contained 8-mer BamHI Tinkers; D221/234+134/1 lx3 contains an insertion of the fragment 7590-7673 in three copies and D221/234+134/11x6 in six copies. D221/234+10BS9 has an insertion of the E2 protein binding site 9 in ten copies. DHindIII/221+10BS9 is a deletion between nucleotides 6959/7892 which carries 10 copies of oligomerized E2 binding site 9. All deletion and insertion mutants were verified by sequencing.

Construction of cell lines. CHO-K1 (Chinese Hamster Ovary - ATCC CCL 61) was used as the parental cell line to express BPV-1 replication proteins.

(i) E2 cell line CHO49. The E2 expression vector pHSE2 was linearized with XhoI and the plasmid pBJ5GS carrying glutamine synthetase minigene expression unit (Bebbington and Hentschel 1987). pBJ5GS (kind gift of Dr. L.Berg) was linearized with SalI endonuclease. The plasmids were mixed at a 1:1 ratio and ligated into the concatemers at high DNA concentration (300μg/ml) overnight at 16^0C using T4 DNA ligase.

Ten micrograms of the ligated DNA was mixed with 5Oμg carrier DNA and was electroporated into the $7x10^6$ CHO-K1 cells using Ham's F12 medium supplemented with 10% fetal bovine serum at 22OV, using a BioRad electroporation apparatus at the capacitance setting 960μF. Selection for glutamine synthetase was done at 25μM concentration of the L-methionine sulfoximine (Sigma) in glutamine free Glasgow Minimal Medium supplemented with dialyzed fetal bovine serum, non-essential amino acids, glutamic acid, aspartic acid, sodium pyruvate, nucleosides, penicillin and streptomycin, essentially as has been described by Bebbington and Hentschel (Bebbington and Hentschel, 1987). Colonies were picked ten days after the selection, expanded and used for the second round of selection at 250 μM of L-methionine sulfoximine. This step was included to amplify the sequences coupled to the selection marker. Cell lines were expanded and tested for expression of E2 protein by immunoprecipitation with polyclonal rabbit antibodies against E2 protein (Ustav and Stenlund, 1991) after labeling with ^{35}S L-methionine using Translabel (ICN) and by functional transient replication assay as described below.

(ii) E1 cell line CH0212. E1 protein expression vector pCGEag (Ustav and Stenlund 1991) was linearized by the XhoI and pBJ5 GS was linearized, mixed at a 1:1 ratio and ligated into the concatemers and the cell line expressing E1 protein was generated essentially same way like E2 expressing cell line.

(iii) E1 and E2 expressing cell line CHO4.15. E1 protein expression vector pE1-lx5 containing glutamine synthetase minigene and E1 coding sequence was linearized with SalI and pHSE2 was linearized with the XhoI restriction endonuclease. Linear plasmids were ligated into the concatemers at a ratio 1:1, CHO-K1 cell were transfected by electroporation and selected as described above.

Transient replication assays were done as described earlier (Ustav and Stenlund, 1991) using the respective cell lines. For testing the cell lines in functional assay for expression of the BPV-1 replication proteins we used 50 ng of pUC/Alu (Ustav *et al.*, 1991) as origin containing plasmid and 250 ng of the E1 expression vector pE1-1x5 or pCGE2 to complement E2 cell line CHO49 or E1 cell line CHO212, respectively. All pNeo5' based origin plasmids were tested for their ability to replicate in the CHO4.15 cell line by transfecting 100 ng of the plasmid DNA together with 50ptg of denatured carrier salmon sperm DNA into the CHO4.15 cells at 24OV by electroporation. Extrachromosomal DNA was extracted from the cells at 48 and 72 hours post-transfection by alkaline lysis as described earlier (Ustav and Stenlund, 199 1). DNA was purified, digested with DpnI and linearizing enzyme and were analyzed by Southern analysis. Specific probes for hybridization were made by random priming.

Stable replication. CHO4.15 cells were electroporated with 100ng of origin containing plasmid DNA in the presence of 5Oμg of carrier DNA. Ninety six hours after transfection CHO4.15 cells were trypsinized and subjected for the antibiotic G418 selection at the concentration 450 μ/ml. Colonies were pooled or single colonies were picked after two weeks, expanded and episomal or total DNA were analyzed by Southern blotting.

Copy-number measurement. Total DNA from established cell lines, containing replicating BPV-1 origin plasmids, was extracted and digested either with a plasmid single-cutter (HindIII) or a plasmid non-cutter (ApaI), followed by electrophoresis in 0.7% agarose/TAE gels. The copynumber was measured by Southern blotting, using probe containing sequences from the BPV ori and neo gene. Results were quantitated by comparing band intensity to a two-fold dilution series of plasmid DNA using phosphoimager.

BrdU labeling and analysis of the replication mode. Cells were labeled with 35 μg/ml BrdU in MEM medium, containing 2'-deoxycytidine (20 μg/ml) using procedures described earlier

(Yates and Guan, 1991). Episomal and chromosomal DNA was extracted at time points indicated in Results section. CsCl solution was added to DNA preparation to 1.74 g/ml and centrifuged for 48 hours at 37000 rpm. 24 fractions were collected from each gradient, subjected to denaturation and neutralization, and slot-blotted onto nylon filters. Filters were hybridized with labeled BPV-1 or CHO genomic DNA probes. Radioactivity was counted with Phosphoimager, Fuji.

Nuclear retention measurement by stable transformation assay. 48 hours following transfection the cells were trypsinized, counted and plated at 3 different dilutions. G418 selection was applied, and the selective medium was changed every 3 days. Colonies were counted after 10 days.

TREATMENT OF DISEASES ACCORDING TO THE INVENTION

The invention is useful in vivo and ex vivo human gene therapy where correction of inherited or acquired genetic defects is desired, and is therefore useful to treat any disease where gene delivery provides benefit, whether the gene is delivered to a terminally differentiated host cell, such as a hepatocyte, or to an undifferentiated cell such as a stem cell. The invention is useful in treatment of chronic or acute diseases, e.g., T-cell diseases, inflammation, fibroses of the liver, and arthritis. The invention also is useful in vaccination protocols where resistance or immunity to infectious pathogens, such as HIV, Hepatitis C Virus, Hepatitis B virus, is desired, or the elimination or induced quiescence of aberrant cells, such as cancer cells, is considered beneficial.

Recombinant vectors of the invention are useful in that they permit persistent expression of a therapeutic gene in both dividing and non-dividing cells; for example, in differentiated cells, such as those in gut, brain, and muscle.

Recombinant vectors of the invention are also useful for high level transient expression in cells where desired, such as for cancer therapy or in vivo vaccination.

Both in vivo and ex vivo gene therapy strategies are possible with this vector system, including stable, multicopy gene maintenance and expression, in haemopoietic and other stem cells, and in the committed and differentiated progeny of these cell types.

Nucleic acid vectors suitable for use in the present invention include circular and linear lengths of DNA.

For human gene therapy, uses of the recombinant vectors of the invention are not limited in terms of delivery of the vector to a cell. That is, vectors of the invention may be delivered to a

cell via non-viral or viral delivery systems. Delivery systems of non-viral origin include those which employ molecular conjugates, cationic liposomes, or synthetic peptides, where vector size constraints do not limit the nature and number of plasmid vector components. Delivery systems of viral origin include viral particle-producing packaging cell lines as transfection recipients for the above E1/E2/M0/MME-containing plasmids into which viral packaging signals have been engineered, such as those of adenovirus, herpes viruses and papovaviruses.

Recombinant vectors of the invention also are useful in transgenesis, including production of transgenic animals via pronuclear injection, or embryonic stem cell transfection and embryo chimera generation.

Heterologous Genes Useful According to the Invention

Heterologous genes useful in vectors of the invention may also encode antigenic determinants of viruses, so as to be useful as vaccines, such antigenic determinants as are present in coat proteins of flu viruses, malaria, TB, and HIV. For modulation of physiologic activity in brain cells, genes including the following may be of interest: CCKA, CCKB, CREB, TH, NT3, NT4, BDNF, GDNF, and NGF.

Regulatory Sequences Useful According to the Invention

As described below, regulatory sequences which may be carried in vectors of the invention will of course include heterologous promoter and enhancer sequences which control expression of the heterologous gene of interest, and which may be confer a tight level of regulation upon the heterologous gene, including inhibition or activation of gene expression, promoter/enhancer strength, tissue specificity, or relatively little regulation beyond the initiation of transcription.

Vectors useful according to the invention may advantageously include a gene encoding a therapeutic agent, a promoter which directs expression of the gene, and optionally a cellular-derived gene regulatory element which confers tissue specific gene expression. The promoter/gene combination may be subject to any one of numerous forms of gene regulation known in the art, for example, production of the gene product may be subject to continuous inhibition by associated factors and thus may require the presence of an activator; alternatively, the gene product may be continuously expressed, and only inhibited under certain conditions. Gene expression may be regulated at either the transcriptional or translational level. Where such

regulation is transcriptional, it may be at the level of the promoter or at the level of RNA elongation or processing. Therefore, vectors useful according to the invention may include a heterologous promoter/gene combination that is turned-on and turned-off in trans by the presence or absence of a regulatory factor.

Heterologous Regulatory Sequences for E1 and E2 Gene Expression

In addition to heterologous sequences to regulate a heterologous gene of interest which is carried on the episomal vector, heterologous regulatory sequences are also used in the invention to control expression of the papilloma E1 and E2 genes. Proper control of expression of E1 and E2 is critical for determining plasmid copy number, stability and segregation, and therefore the invention encompasses maintenance of an MO+MME-containing plasmid in eukaryotic cells. The presence of heterologous regulatory elements has been found to influence persistent expression, expression in different cell types, and expression in vivo.

Choice of a heterologous promoter to drive E1 gene expression has been found to confer certain advantages to a vector of the invention, depending upon the intended use of the construct. For example, it has been found that a strong promoter such as SRalpha (a hybrid of SV40 early region and HTLV-1 LTR) to drive expression of the E1 gene can be used advantageously according to the invention to drive plasmid copy number very high, that is, to levels which render the host cell unhealthy and prevent normal cell division. This generates unstable, onion skin-like products and leads to cell inviability (Figure 10). However, a strong promoter is advantageously used where high level transient expression of a vector heterologous gene is desired, for example, in treatment of a cancerous condition where it is desirable to produce a high level of heterologous gene product (for example, a toxin) and it is also desirable to kill the host cell (for example, tumor cells). This type of vector of the invention takes advantage of the ability of the MO/MME vector system to replicate at very high levels independently of the cell cycle.

In contrast, it has also been found that use of a weaker promoter, such as the thymidine kinase promoter, to drive E1 gene expression can be used advantageously in the invention to permit sufficient E1 protein expression to regulate stable (MO+MME)-containing plasmid replication at high efficiency.(Figure 10). Similarly, use of the weaker promoter such as thymidine kinase promoter permits E1 protein synthesis to a level compatible with efficient expression of a reporter gene product from the constructs in transient assay (Figure 11). The results shown in Figure 12 demonstrate stable expression of beta-gal in the cells upon selection.

SRalpha-E1 constructs express reporter, however cells are not dividing while Tk-E1 constructs work for expression in transient as well as in stable cells; cells divide normally to give colonies which stain blue for beta-gal protein.

Proper control of post-transcriptional processing of E1 and E2 transcripts further influences the replicative and expression properties of the plasmid system (efficiency, compatibility with host replication system, stability, toxicity), and is improved by incorporation of sequences such as those of human beta globin gene (exxonII, intronII, exonIII sequences), and SV40 sequences which incorporate splice, polyA and mRNA stability sequences. Improved expression vectors harboring such elements in the E1 and E2 expression domains are described herein. Figs. 13A and 13B show G418 selection of different vectors (SRalpha-E1 and TkE1 constructs).

In addition to the use of regulatory sequences for regulation of E1 and E2 gene expression, the invention also encompasses the use of regulatory sequences for control of a heterologous gene encoding a protein of interest, which gene is carried on an episomal vector of the invention.

The adenovirus E1A promoter and enhancer, the human CMV-MIE promoter and enhancer, retroviral LTR elements, herpesvirus promoters, and poxvirus promoters are representative examples of heterologous regulatory elements useful in the invention.

Modification of a heterologous promoter useful according to the invention may be accomplished according to a number of strategies. For example, the use of negative regulatory elements which decrease the level of transcription is envisaged. Especially preferred, however, is the modification of the promoter sequences in order to reduce the basal levels of transcription. Promoter sequences may be modified in order to remove regulatory elements which respond to cell-specific regulatory factors. Preferably, therefore, elements responsible for activation by cell-specific factors may be mutated or deleted.

Modified Forms of a Promoter Useful in the Invention

The invention encompasses the use of modified forms of a heterologous promoter, which exhibit decreased levels of basal transcription. Such heterologous promoters may be useful in the invention, because it may be desirable to confer upon the host cell a low enough level of transcription of, for example, an anti-viral gene or a gene encoding a toxin so as to prevent

deleterious effects on the host cell by virtue of the presence of the encoded gene product in the cell.

Modified forms of a given promoter may be made, as is well-known in the art, using conventional PCR and incorporating random or directed base substitutions, deletions, or insertions in the native promoter sequence.

Transactivatable viral promoters useful according to the invention include but are not limited to the following: Herpes Virus (HSV-1), immediate early promoter (Liv *et al.*, 1990, Biotechniques 9:168; Rice *et al.*, 1990, Jour. Virol. 64:1704; Howard *et al.*, 1993, Exp. Cell. Res. 207:194); Visna Virus promoters (Carroth *et al.*, 1994, Jour. Virol. 68:6137); Papillomavirus promoters (Storey *et al.*, 1990, Jour. Ge. Virol. 71-965 and Ibaraki *et al.*, 1993, Virus Genes 7:187; Epstein-Barr Virus promoters (TPI and Barn HIC promoters) Cohen *et al.*, 1991, Jour. Virol. 65-5880; Sung *et al.*, 1991 Jour. Virol. 65:2164; and Meitinger *et al.*, 1994, Jour. Virol. 68:7497); CMV promoters (IE2, IE1, and MIE promoters) (Malone *et al.*, 1990, Jour. Virol. 64:1498; Cockett *et al.*, 199 1, Nucl. Ac. Res. 19:319; and Pizzomo *et al.*, 1991, Jour. Virol. 65:3839); Hepatitis B promoters (Seto *et al.*, 1990, Nature 344:72; Raney *et al.*, 1991, Jour. Virol. 65:5774; Lauer *et al.*, 1994, Hepatology 19:23); Spumaretroviral promoters (Rethwillm *et al.*, 1990, Virol. 175:568, Venkatesn *et al.*, 1993, Jour. Virol. 67:3868); HTLV-1 promoters (Franklin *et al.*, 1993, Jour. Biol. Chem. 268:21225; Kadison *et al.*, 1990, Jour. Virol. 64:2141); and Adenoviral promoters (E2) (Obert *et al.*, 1994, Mol. All. Biol. 14:1333).

Heterologous promoters useful according to the invention, including modified forms of a given promoter, are first tested for basal activity using a reporter gene, and are also tested to determine if they possess or retain the ability to be trans-activated. Where a vector of the invention includes heterologous promoter/gene combination which is present in the cell in an inhibited state and which requires transactivation for production of the encoded heterologous gene product, such promoter/gene combinations may be most useful provided they possess or retain the ability to be transactivated while exhibiting a low enough level of basal activity such that the gene product is virtually undetectable.

A vector of the invention, whether its heterologous promoter is modified or unmodified, may be tested for its ability to be trans-activated to the extent that differential killing of transformed cells occurs. Functional tests of diminished basal activity from vectors of the invention will include the use of prodrug activating systems, such as ganciclovir, in cells containing a vector in which the heterologous promoter/gene combination is in the inhibited versus the activated state.

Regulatory Elements conferring Cell-Type Specific Heterologous Gene Expression

Cellular-derived genetic elements also may be included in episomal vectors of the invention.

These genetic elements thus are responsive to, i.e., subject to control by, cellular factors. Tissue-specific promoters may confer tissue specificity.

E2 Mutants Defective for Transcriptional Activation

The invention also encompasses the use of E2 mutants which are defective in the E2 transcriptional activator function. A gene encoding such an E2 mutant is incorporated into the episomal vector system of the invention.

Full-length E2 protein has two functional domains which are well-conserved among the E2 proteins of different papollomaviruses, a 200 amino acid transactivation domain at the amino terminus and a 90-100 amino acid carboxy terminal domain that is essential for dimerization and DNA binding. A flexible spacer hinge region separates the transactivation and DNA binding domains.

In order to provide an E2 mutant which is defective in transcriptional activation, but which are able to supply the E2 function that is necessary and sufficient for episomal persistence, point mutations have been made in the gene encoding this protein. These mutations are useful where they eliminate the transcriptional activating activity of the protein without affecting the ability of the resultant protein to stably maintain (MO+MME)-containing plasmids in transfected cells.

E2 mutants were made by selected mutation of amino acids which are believed to lie in hydrophilic domains at the protein surface, and by then changing basic or acidic amino acids to a small hydrophobic and/or polar residue, e.g., alanine, which has a short side chain consisting of a single -CH_3 group; serine, which also contains a single carbon atom side chain (-CH_2OH); valine, leucine, threonine and isoleucine, which are hydrophobic amino acids having side chains ranging from 2-4 carbon-containing groups. The mutation is introduced into the E2 gene using oligonucleotide-directed mutagenesis in M13 phage. It is within the scope of the invention to locate additional point mutations which abolish the transcription activating activity of E2 without deleteriously affecting its replication function to maintain episomal vectors according to the invention.

Manufacture of Vectors of the Invention

The invention also features vectors which include sequences conferring replication and selection in lower eukaryotic or prokaryotic host cells in order to manufacture a useful quantity of vector DNA, e.g., 100ug - mg quantities.

Additional sequences which may be present in a vector of the invention to enable manufacture of vector DNA include other origins of replication, e.g., a bacterial or yeast origin of replication, which permits preparation of vector DNA in prokaryotic or eukaryotic cells, or baculovirus sequences which allow for vector replication in insect cells.

This aspect of the invention is applicable to most strains of bacteria, for example, gram positive and negative bacterial strains, and yeast. Gram negative bacteria useful according to the invention include but are not limited to E. coli and Salmonella, e.g., S. typhimurium. Gram positive species useful according to the invention include but are not limited to Bacillus and Lactococcus.

Prokaryotic and Eukaryotic Origins of Replication

The invention can be utilized advantageously with a plasmid origin of replication that permits replication of at least 10, preferably at least 20-100, and most preferably at least 200-500 copies of the plasmid per host cell. Those origins of replication that permit replication of moderate (i.e., 2050) to high plasmid (i.e., 200-500) copy numbers are especially useful. Of course, if desired, a plasmid having a copy number, as high as 1000-2000 copies per cell, also may be used.

Plasmids with low copy numbers (i.e., 10 copies or less) are most advantageously used according to the invention after mutation to bring about increased copy number (J. Scott, 1984, Microbial Reviews 48:1-23). Of the frequently used origins of replication, pBR322 (20 copies/cell) is useful according to the invention, as is pUC (at 200 copies/cell). Other plasmids whose origins of replication may be useful according to the invention are those which require the presence of plasmid encoded proteins for replication, for example, the pT181, FII, and FI origins of replication.

Examples of origins of replication which are useful according to the invention in E. coli and S. typhimurium include but are not limited to pMB1 (25 or more copies per cell, Bolivar *et*

al., 1977, Gene 2:95-113), ColE1 (15 or more copies per cell, Kahn *et al.*, 1979, Methods Enzymol. 68:268280), p15A (about 15 copies per cell, Chang *et al.*, 1978, J. Bacteriol. 134:1141-1156); pSC101 (about 6 copies per cell, Stoker *et al.*, 1982, Gene 18:335-341); R6K (less than 15 copies per cell, Kahn et al, 1979, supra); R1 (temperature dependent origin of replication, Uhlin et al, 1983, Gene 22:255-265); lambda dv (Jackson *et al.*, 1972, Proc. Nat. Aca. Sci. 69:2904-2909). An example of an origin of replication that is useful in Staphylococcus is pT181 (about 20 copies per cell, J. Scott, 1984, Microbial Reviews 48:1-23. Of the above-described origins of replication, pMB1, pl5A and ColE1 are preferred because these origins do not require plasmid-encoded proteins for replication.

Genes Encoding Selectable Marker Useful in Vectors of the Invention

Genes encoding selectable markers useful in vectors of the invention include antibiotic resistance genes, for example encoding resistance to antibiotics such as ampicillin, kanamycin or tetracycline, are the most common dominant selectable markers used in molecular biology cloning and fermentation procedures for the production of recombinant proteins or plasmid DNA.

Preparation of vector DNA can also be used in conjunction with antibiotic resistance. Representative antibiotic resistance genes useful according to the invention include, for example, the kanamycin gene, from pUC4K (Pharmacia Biotech) by restricting the plasmid with XhoI and filling in the 5' overhang. This plasmid DNA is then restricted with PstI and the fragment containing the kanamycin gene is then gel purified. Other useful antibiotic resistance genes are well-known in the art, including the genes encoding chlorainphenicol acetyl transferase, ampicillin, and tetracycline.

A vector of the invention which is intended for manufacture in bacteria will include in addition to the papillomavirus replication elements described herein, for example, a bacterial origin of replication and a gene encoding a selectable marker. The vector DNA will be transformed into a bacterial host, and the host grown under selection conditions. Plasmid DNA is then prepared according to conventional means.

Delivery of Episomal Vector to Host Cell

Vectors of the invention may be delivered to a host cell via any one of a number of vector delivery means. The invention is not limited by the mode of delivery of the vector to the host cell.

Transfer of a vector of the invention to a host cell can be accomplished via any of the following, including but not limited to direct injection of naked DNA, for example, using a gene gun, transfection using calcium phosphate coprecipitation, fusion of the target cell with a liposomal vehicle, erythrocyte ghosts or spheroplasts carrying DNA, plasmid and viral vector-mediated transfer, DNA protein complex-mediated gene transfer such as receptor-mediated gene transfer, and viral infection.

Receptor-mediated gene transfer is dependent upon the presence of suitable ligands on the surfaces of cells which will allow specific targeting to the desired cell type followed by internalization of the complex and expression of the DNA. One form of receptor-mediated gene transfer is wherein a DNA vector is conjugated to antibodies which target with a high degree of specificity cell-surface antigens (Wong and Huang, 1987, Proc. Nat. Aca. Sci. 84:7851; Roux *et al.*, 1989, Proc. Nat. Aca. Sci. 86::9079; Trubetskoy *et al.*, 1992, Bioconjugate Chem. 3:323; and Hirsch *et al.*, 1993, Transplant Proceedings 25:138). Nucleic acid may be attached to antibody molecules using polylysine (Wagner *et al.*, 1990, Proc. Nat. Aca. Sci. 87:3410; Wagner *et al.*, 1991, Proc. Nat. Aca. Sci. 89:7934) or via liposomes.

Increased expression of DNA derived from ligand-DNA complexes taken up by cells via an endosomal route has been achieved through the inclusion of endosomal disruption agents, such as influenza virus hemagglutinin fusogenic peptides, either in the targeting complex or in the medium surrounding the target cell. The fusogenic peptide of the HA molecule is a modified form of HA which retains two important functions of HA. It allows for fusion of the targeted DNA/ligand complex to the cell membrane, but without the host cell sialic acid-binding specificity of the natural molecule. Instead, host cell binding specificity is conferred by the ligand/receptor interaction. The modified HA fusogenic peptide also retains the HA function of endosomal uptake, thus allowing for uptake of the complex into the host cell via membrane fusion, and the endosomal escape function of HA, which allows for escape of the enveloped DNA from the endosomal/lysosomal destruction pathway. The fusogenic peptide may include the HA amino acid sequence GLFGAIAGFIGAGTGGMIAGGGC.

1. Viral Vectors

Recombinant viral vectors as well as other DNA transfer schemes can be used in practice of the present invention. A recombinant viral vector of the invention will include DNA of at least a portion of a viral genome which portion is capable of infecting the target cells. By "infection" is

generally meant the process by which a virus transfers genetic material to its host or target cell. Preferably, the virus used in the construction of a vector of the invention is also rendered replication-defective to remove the effects of viral replication on the target cells. In such cases, the replication-defective viral genome can be packaged by a helper virus in accordance with conventional techniques. Generally, any virus meeting the above criteria of infectiousness and capabilities of functional gene transfer can be employed in the practice of the invention.

Suitable viruses for practice of the invention include but are not limited to, for example, papovavirus and herpesvirus, well known to those skilled in the art; suitable vector packaging cell lines for stably transducing mammalian cell lines are known in the art.

It will be appreciated that when viral vector schemes are employed for gene transfer according to the invention, the use of an attenuated or a virulent virus also may be desirable.

2. Delivery of Gene via DNA-Protein Complexes

Targeted gene delivery is also achieved according to the invention using a DNA-protein complex. Such DNA-protein complexes include DNA complexes with a ligand that interacts with a target cell surface receptor. Cell surface receptors are thus utilized as naturally existing entry mechanisms for the specific delivery of genes to selected mammalian cells. It is known that most, if not all, mammalian cells possess cell surface binding sites or receptors that recognize, bind and internalize specific biological molecules, i.e., ligands. These molecules, once recognized and bound by the receptors, can be internalized within the target cells within membrane-limited vesicles via receptor-mediated endocytosis. Examples of such ligands include but are not limited to proteins having functional groups that are exposed sufficiently to be recognized by the cell receptors. The particular proteins used will vary with the target cell.

Typically, glycoproteins having exposed terminal carbohydrate groups are used although other ligands such as antibodies or polypeptide hormones, also may be employed. Using this technique the phototoxic protein psoralen has been conjugated to insulin and internalized by the insulin receptor endocytotic pathway (Gasparro, Bio-chem. Biophys. Res. Comm. 141(2), pp. 502509, Dec. 15, 1986); the hepatocyte specific receptor for galactose terminal asialoglycoproteins has been utilized for the hepatocyte-specific transmembrane delivery of asialoorosomucoid-poly-L-lysine non-covalently complexed to a DNA plasmid (Wu, G.Y., J. Biol. Chem., 262(10), pp. 44294432, 1987); the cell receptor for epidermal growth factor has been utilized to deliver polynucleotides covalently linked to EGF to the cell interior (Myers,

European Patent Application 86810614.7, published Jun. 6, 1988); the intestinally situated cellular receptor for the organometallic vitamin B_{12}-intrinsic factor complex has been used to mediate delivery to the circulatory system of a vertebrate host a drug, hormone, bioactive peptide or immunogen complexed with vitamin B_{12} and delivered to the intestine through oral administration (Russel-Jones *et al.*, European patent Application 863 07849.9, published Apr. 29, 1987); the mannose-6-phosphate receptor has been used to deliver low density lipoproteins to cells (Murray, G. J. and Neville, D. M., Jr., J. Bio. Chem. Vol. 225 (24), pp. 1194-11948, 1980); the cholera toxin binding subunit receptor has been used to deliver insulin to cells lacking insulin receptors (Roth and Maddox, J. Cell. Phys. Vol. 115, p. 151, 1983); and the human chorionic gonadotropin receptor has been employed to deliver a ricin a-chain coupled to HCG to cells with the appropriate HCG receptor in order to kill the cells (Oeltmann and Heath, J. Biol. Chem, Vol 254, p. 1028 (1979)). Ligands selected from biotin, biotin analogs and biotin receptor-binding ligands, and/or folic acid, folate analogs and folate receptor-binding ligands to initiate receptor mediated transmembrane transport of the ligand complex, as described in U.S. Pat. No. 5,108,921.

Generally, a ligand is chemically conjugated by covalent, ionic or hydrogen bonding to the nucleic acid. A ligand for a cell surface receptor may be conjugated to a polycation such as polylysine with ethylidene diamino carbodiimide as described in U.S. Patent No. 5,166,320. DNA may be attached to an appropriate ligand in such a way that the combination thereof or complex remains soluble, is recognized by the receptor and is internalized by the cell. The DNA is carried along with the ligand into the cell, and is then expressed in the cell. The protein conjugate is complexed to DNA of a transfection vector by mixing equal mass quantities of protein conjugate and DNA in 0.25 molar sodium chloride. The DNA/protein complex is taken up by cells and the gene is expressed.

Delivery of the foreign DNA into the target cell may also be achieved via the DNA construct's association with an endosomal disruption agent, such as the influenza hemagglutinin fusogenic peptide, as described above.

3. Liposomal Gene Transfer

Liposomes have been used for non-viral delivery of many substances, including nucleic acids, viral particles, and drugs. A number of reviews have described studies of liposome production methodology and properties, their use as carriers for therapeutic agents and their

interaction with a variety of cell types. See, for example, "Liposomes as Drug Carriers," Wiley and Sons, NY (1988), and "Liposomes from Biophysics to Therapeutics," Marcel Dekker, NY (1987). Several methods have been used for liposomal delivery of DNA into cells, including poly-L-lysine conjugated lipids (Zhou *et al.*, Biochim. Biophys. Acta. 1065:8-14, 1991), pH sensitive immunoliposomes (Gregoriadis, G., Liposome Technology, Vol I, II, III, CRC, 1993), and cationic liposomes (Felgner *et al.*, Proc. Natl. Acad. Sci., USA, 84:7413-7417, 1987). Positively charged liposomes have been used for transfer of heterologous genes into eukaryotic cells (Felgner *et al.*, 1987, Proc. Nat. Aca. Sci. 84:7413; Rose *et al.*, 1991, BioTechniques 10:520). Cationic liposomes spontaneously complex with plasmid DNA or RNA in solution and facilitate fusion of the complex with cells in culture, resulting in delivery of nucleic acid to the cell. Philip *et al.* 1994, Mol. and Cell. Biol. 14:2411, report the use of cationic liposomes to facilitate adeno-associated virus (AAV) plasmid transfection of primary T lymphocytes and cultured tumor cells.

Delivery of an agent using liposomes allows for non-invasive treatment of diseases. Targeting of an organ or tissue type may be made more efficient using immunoliposomes, i.e., liposomes which are conjugated to an antibody specific for an organ-specific or tissue-specific antigen. Thus, one approach to targeted DNA delivery is the use of loaded liposomes that have been made target-specific by incorporation of specific antibodies on the liposome surface.

Host Cells Useful in the Invention

The cells targeted for in vivo or ex vivo gene transfer in accordance with the invention include any cells to which the delivery of the therapeutic gene is desired. Eukaryotic cells are preferred, and particularly mammalian cells. For example, brain cells, cells of the central nervous system, nerve cells, gut cells, skin cells, kidney cells, endothelium, lung cells, liver cells, cells of the immune system such as T-cells, B-cells, and macrophages.

Dosage, Mode of Administration and Pharmaceutical Formulation

Vector DNA may be formulated as naked DNA for parenteral administration or as a transfection mixture. In the latter case, the transfection mixture may be assembled just prior to use. In the case of a pharmaceutical composition, the vector DNA includes the papillomavirus MO and MME sequences, optionally, the E1 and E2 genes, and a gene whose expression is intended to have some beneficial therapeutic effect on the cells of the recipient. For optimal

efficiency of delivery of naked DNA to a target tissue, it is preferred that the vector DNA be delivered at 10-100ug/10,000 cells, optimally about 50ug/10,000 cells.

The DNA may be exchanged into isotonic phosphate free buffer and sterile filtered, and then aliquotted into suitable vials. The vials may be stored at 4°C, 20°C or 80°C or alternatively the mixture may be freeze dried from a buffer containing an appropriate carrier and bulking agent. In these cases, the dosage form is reconstituted with a sterile solution before administration.

For delivery of vector DNA in vivo or ex vivo, the vector containing a gene of physiological importance, such as replacement of a defective gene or an additional potentially beneficial gene function, is expected to confer long term genetic modification of the cells and be effective in the treatment of disease.

For example, a patient that is subject to a viral or genetic disease, or who is being vaccinated against a virus or pathogen, may be treated in accordance with the invention via in vivo or ex vivo methods. For example in vivo treatments, a vector of the invention can be administered to the patient, preferably in a biologically compatible solution or a pharmaceutically acceptable delivery vehicle, by ingestion, injection, inhalation or any number of other methods. The dosages administered will vary from gene to gene, disease to disease, and from patient to patient; a "therapeutically effective dose" will be determined by the level of enhancement of function of the transferred genetic material balanced against any risk or deleterious side effects. Monitoring levels of gene introduction, gene expression and/or the presence or levels of the encoded therapeutic protein will assist in selecting and adjusting the dosages administered. Generally, a composition including the vector will be administered in a single dose in the range of 10 ng - 1 mg/kg body weight, preferably in the range of 100 ng - 100 ug/kg body weight, such that at least one copy of the therapeutic gene is delivered to each target cell. Vector DNA may be administered as a single dose, or in multiple doses, as needed. The therapeutic gene will, of course, be associated with appropriate regulatory sequences for expression of the gene in the target cell.

Ex vivo treatment is also contemplated within the present invention. Cell populations can be removed from the patient or otherwise provided, transduced with a therapeutic gene in accordance with the invention, then reintroduced into the patient. In general, ex vivo cell dosages will be determined according to the desired therapeutic effect balanced against any deleterious side-effects. Such dosages will usually be in the range of 10^5- 10^8 cells per patient, daily weekly, or intermittently; preferably 10^6- 10^7 cells per patient.

EXAMPLES

The following examples describe detailed embodiments of the invention in which a given gene of interest is introduced and stably maintained in a specific host cell, or a specific disease is exemplified for treatment according to the invention.

EXAMPLE 1

Recombinant Host Cells of the Invention

Recombinant vectors of the invention may be used in the in vitro production of a protein of interest, for example, cell factories may be prepared by transforming a host cell with a recombinant vector of the invention. The vector will contain the papillomavirus MO, MME and a gene encoding the protein of interest, and may optionally include the genes encoding the HPV or BPV E1 and E2 genes. Alternatively, a cell line may be prepared that carries in its chromosome the E1 and E2 genes, such that the encoded proteins are in trans to the papilloma episomal sequences. A cell line carrying a recombinant vector of the invention, whether it carries the E1 and E2 genes in cis or in trans to the vector permits stable, high level expression of proteins of therapeutic value in cultured mammalian cells.

Where it is desirable according to the invention to determine the copy number (per haploid or diploid host cell genome) of a recombinant vector of the invention. Vector copy number may be determined as described herein for copy number determination of the BPV origin-containing plasmids.

Described below is an example in which a host cell line is engineered to contain chromosomal copies of the E1 and E2 genes. This cell line is advantageous for production of a desired protein or RNA in that it can be transfected with a vector of the invention encoding the desired protein or RNA, and cultured to produce that molecule.

We constructed several cell lines constitutively expressing the E1 and E2 proteins. Expression of these proteins was directed from integrated constructs for E1 protein from CMV promoter (cell line CHO212) and for E2 protein from HAP70 promoter (cell line CHO49). In the cell line CHO4.15 which expresses both E1 and E2, the E1 protein was expressed from SRαpromoter and the E2 protein from HSP70 promoter. Selection of the respective cell lines and amplification of the expression units of interest was achieved by utilizing the glutamine synthetase

minigene from the pSVLGS.1 plasmid according to the protocol described earlier (Bebbington and Hentschel, 1987). Expression of E1 and E2 was identified by immunoprecipitation using specific rabbit polyclonal sera (data not shown) and by in vivo replication assays. The three cell lines and the parental CHO cells were transfected with the BPV-1 origin containing plasmid pUC/Alu in combination with E1 and E2 expression vectors. The cell line CHO4.15 which expresses both E1 and E2, supports replication of the origin plasmids in the absence of erogenous E1 and E2. The E2 expressing cell line, CHO49, supports replication in the presence of an E1 expression vector, but fails to do so without erogenous E1. The E1 expressing cell line, CHO212, supports replication only in the presence of an E2 expression vector. In the parental CHO cell line, co-expression of both E1 and E2 is required for replication. No replication of pUC/Alu can be detected in the absence of E1 and E2.

The cell line CHO4.15 is transformed with a vector of the invention, for example, the pBN/TKE1-0 vector shown in detail in Fig. 7B. Alternatively, where transient high level expression of the gene of interest is desired, the vector pBNE1, shown in Fig. 7A may be used to produce relatively higher quantities of the protein of interest. We have observed MO/MME vector persistence in CHO4.15 cells for at least 80 cell generations with a copy number loss less than 10% over 80 generations. Each of these vectors contains the beta-galactosidase reporter gene. However, as is described below, the beta-gal gene can be removed and substituted with a gene of interest. The transformed cells are grown under conditions, as described in Materials and Methods, to produce the encoded molecule of interest.

EXAMPLE 2

E2 Transcriptional Activator Mutants

The invention also encompasses the use of E2 point mutants which are defective in the E2 transcriptional activator function and competent in the replication function. Such E2 mutants are incorporated into the episomal vector system of the invention. Point mutations have been made in the gene encoding this protein which eliminate the transcriptional activating activity of the protein without affecting the ability of the resultant protein to stably maintain (MO+MME)-containing plasmids in transfected cells.

The mutants were made by selected mutation of amino acids which are believed to lie in hydrophilic domains (i.e., hydrophilic domains of between about 4-15 amino acids) and/or alpha

helical domains at the protein surface. Such mutants are made, for example, by amino acid substitutions in the alpha helix region 2 (amino acid residues 23-49) and helix 3 (amino acids residues 62-82), and by then changing basic or acidic amino acids to a small hydrophobic residue. The mutation is introduced into the E2 gene using oligonucleotide-directed mutagenesis in M 13 phage. It is within the scope of the invention to locate additional point mutations which abolish the transcription activating activity of E2 without deleteriously affecting its replication function.

Figure 14A depicts the location of the mutations and schematically illustrates the predicted secondary structure (cylinder - -helix, shaded box - -sheet) for the N-terminal and the X-ray structure for the C-terminal portions of the E2 protein. The locations of mutations are indicated by arrows; numbers refer to the position of the mutated residue in the E2 protein.

Figure 14B demonstrates that mutant E2 proteins are stably expressed in cells. Immunoblots of the E2 proteins are shown. Cell lysates were prepared from COS-7 cells containing a wild-type (WT) or mutant E2 expression plasmid. 30µl of cell lysate was loaded on each lane. The negative control lysate was prepared from cells electroporated with carrier DNA. The substitutions of amino acids are shown on the lettering, and single amino acid code is used; the numbers refer to the positions of the substituted residues in the E2 protein.

Figure 15A shows the replication activation properties of the mutant E2 proteins using transient replication assays of the mutated E2 proteins in CHO cells. Cells were electroporated with 100 ng of reporter plasmid pUC/Alu, 500 ng of pCGEag and 250 ng of pCGE2, which expresses wild-type E2 or its derivatives. Cells were harvested either 36 or 48 hours after electroporation, low mol.wt. DNA was digested with DpnI and linearizing enzyme Hind III and analyzed by the Southern blotting.

Figure 15B shows the reporter constructs for determining transcription activation properties of the E2 mutants, and origin constructs for determining activation of replication. The numbers indicate nucleotide positions in the BPV URR sequence. pUCAlu was used for transient replication studies. pPCAT and pSV3BS9CAT are the CAT reporter plasmids used in transcriptional activation assays.

Figure 16 shows the transcription activation activity of the mutant E2 proteins and represents comparison of transactivation and DNA binding abilities of E2 protein mutants. The radioactive signals of gel-shift assays were quantitated with the use of a PhosphorImager (data not shown). After scanning, the E2 specific signal of wtE2 protein was set as 1. For transcription assay CHO cells were electroporated with 250 ng of respective reporter and 250 ng of pCGE2 or

its derivatives. Normalized CAT activities were determined 40 hours after transfection. In all cases the values shown represent the results of three independent transfection experiments.

Figure 17 demonstrates of the self-squelching feature of some of the E2 mutants at the higher concentrations. Transient transcription assay for the E2 protein mutants were performed as described in Materials and Methods. CHO cells were transfected with increasing amounts of the expression vector for E2 mutants or chimeric E2 proteins, as indicated, and with 250 ng of reporter plasmid pSV3BS9CAT. Normalized CAT activities were determined 40 hours after transfection. Each point represents the result obtained in three independent transfection assays. Fig. A. Mutations with nearly wild-type properties in transient transcription assay. B. Mutations, which transcriptional activity has decreased to 50% of that of the wild-type protein. C. Inactive mutations for transcription. D. Transcriptional properties of chimeric proteins p53:E2 and VP16:E2.

The mutants R37A (i.e., Arg-37-->Ala), E74A (Glutamic Acid-74 -->Ala), D122A, D143A/R172C are particularly useful according to the invention because they are defective or crippled for transcriptional activation of promoters are essentially wild type for activation of replication in the transient replication assay.

Table 1 shows codons which were mutated, giving rise to the described amino acid changes.

Table 1

codon start	wt codon	mutated codon	change	name
2716	AGA	GCA	Arg-37 Ala	R37A
2827	GAA	GCA	Glu-74 Ala	E74A
3121	CGC	TGC	Arg-172 Cys	R172C

E2 mutants such as those described herein can be tested for their ability to replicate MO+MME containing plasmids using the MO/MME replication assays described herein. The use of such E2 mutants is advantageous in that it eliminates, or at least minimizes, the possibility of aberrant activation of cellular genes including proto- oncogenes by the E2 protein, and represents an improved safety feature of this system. The safety of the vector is increased by the inactivation of the E2 transcription activation domain. Furthermore, the specificity of the vector and also its safety is increased because the mutant form of E2 would not activate E2 responsive promoters

which may be present on the vector. In addition, the mutant form of E2 described herein would not transactivate E2 enhancers. It is known that some E2 binding sites constitute an E2 enhancer. These binding sites, when present on a vector according to the invention as part of the MME, would not be transactivated by mutant E2, thus further reducing the deleterious effects of wild-type E2 in this vector system. Use of the mutant form of E2 disclosed herein also may improve the specificity of tissue-specific promoters or other promoters which drive expression of a therapeutic (or other) gene on the vector. That is, because promoters may have some leakiness, transcription from a leaky promoter driving E2 gene expression may result in E2 expression and initiation of E2 transactivation of an E2-driven promoter and gene. However, the inventive mutant form of E2 is defective in transactivation and therefore would not initiate E2-responsive transcription even in the presence of a leaky promoter driving mutant E2 gene expression. Therefore, a tissue-specific promoter which drives expression of a gene of interest on a vector of the invention may be more tightly regulated as a result of use of an E2 mutant according to the invention.

EXAMPLE 3

Stable persistence of Vector of the Invention in Muscle Cells

The usefulness of the E1/E2/M0/MME episomal vector system for in vivo delivery to, and long term expression in, mammalian muscle is demonstrated as follows. Generation of a humoral immune response to genes expressed in these vectors has been achieved by expression in muscle by direct intramuscular injection of vector DNA, testifying to its utility as an in vivo vaccine system. Briefly, the E1/E2/M0/MME plasmid is directly injected into mice intramuscularly into each leg; 50ul saline containing 10Oug plasmid DNA is injected into the quadraceps muscle of each leg. Injections were performed in 1, 2, or 3 sets at 3 week intervals (Ulmer, 1993, Science 259;1745). Fig. 19 shows the results of the experiment. 0.1 mg of plasmid DNA was injected into mice intramuscularly. Serum samples were collected at 90 days after injection. Antibodies were measured by ELISA using recombinant β-galactosidase as antigen. The OD at 414 was read using a 1:1000 dilution of serum. "Neg. Control" refers to saline alone injection; "pUE83" refers to an RSV LTR promoter-driven β-gal expression cartridge cloned into pUC19; " SRalpha vector" refers to the E1/E2/MO/MME plasmid containing the E1 and E2 genes driven by the SRalpha promoter and the same β-gal expression cartridge; and "TK vector" refers to the E1

/E2/M0/MME plasmid containing the E1 and E2 genes driven by the TK promoter and the same β-gal expression cartridge. The MME in the SRalpha and TK vectors is the region in the BPV URR corresponding to positions 6958-1.

EXAMPLE 4

Stable persistence of Vector of the Invention in Brain Cells

The following example demonstrates persistent, long term expression of lacZ reporter gene in the central nervous system using an E1/E2/M0/MME vector harboring a lacZ reporter gene (Figure 18). Briefly, the vector SR-alpha #2 (SR promoter directing E1 and E2 expression in the E1/E2/M0/MME plasmid described in detail above) was injected into an adult rat brain striatum region (bregma +1.7 dex. +2.5). Total amount of vector DNA was 50 micrograms in 5 microliter of PBS. Plasmid DNA was dissolved in PBS (panels E and F) or artificial cerebrospinal fluid (panels A-D). The results showed that P -galactosidase activity was detected histochemically by X-GAL regular staining protocol on frozen brain tissue slices after 5 days (panels A and B), two weeks (panels C and D) and three months (panels E and F) exposure. Control animals were injected with PBS (E) or CSF (panels A and C). Therefore, the plasmid was stably maintained in brain tissue for as long as three months after introduction.

The above example demonstrates the utility of vectors of the invention in gene therapy and in providing animal models of disease and therapy.

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OTHER EMBODIMENTS

Other embodiments will be evident to those of skill in the art. It should be understood that

the foregoing detailed description is provided for clarity only and is merely exemplary. The spirit and scope of the present invention are not limited to the above examples, but are encompassed by the following claims.

Claims

1. A method of obtaining long-term stable production of a gene product of interest in a host cell, comprising

providing a host cell containing a vector comprising

(A) a minimal origin of replication of a papilloma virus,

(B) a minichromosomal maintenance element of a papilloma virus, and

(C) a gene encoding said gene product,

wherein said vector, when present in a mammalian host cell, persists in said cell for at least about 50 cell generations in dividing cells or for at least about 8 weeks in non-dividing cells under nonselective conditions without an appreciable loss of copy number.

2. A method of obtaining long-term stable production of a gene product of interest in a host cell, comprising

providing a host cell containing a vector comprising papillomavirus sequences consisting essentially of

(A) a papillomavirus E1 gene and E2 gene,

(B) a minimal origin of replication of a papilloma virus,

(C) a minichromosomal maintenance element of a papilloma virus, and

(D) a gene encoding said gene product,

wherein said vector persists in said cell for at least about 50 cell generations in dividing cells or for at least about 8 weeks in non-dividing cells under nonselective conditions without an appreciable loss of copy number.

3. A method of obtaining long-term stable production of a gene product of interest in a host cell, comprising

providing a host cell containing a pair of vectors comprising

(I) a first vector comprising papillomavirus sequences consisting essentially of

(A) a papillomavirus E1 and E2 gene,

(B) a minimal origin of replication of a papilloma virus, and

(C) a minichromosomal maintenance element of a papilloma virus, and

(II) a second vector comprising papillomavirus sequences consisting essentially of

(A) a gene encoding said gene product,

(B) a minimal origin of replication of a papilloma virus, and

(C) a minichromosomal maintenance element of a papilloma virus,

wherein said vector persists in said cell for at least about 50 cell generations in dividing cells or for at least about 8 weeks in non-dividing cells under nonselective conditions without an appreciable loss of copy number.

4. Use of a recombinant vector for obtaining long term stable maintenance of erogenous DNA in a eukaryotic host cell wherein the recombinant vector comprises:

minimal origin of replication of a papillomavirus;

minichromosomal maintenance element of a papillomavirus; and

heterologous DNA sequence encoding an expressible gene.

5. The method of claims 1-3 or the use of claim 4, said minichromosomal maintenance element of papillomavirus being from BPV.

6. The method of claims 1-3 or the use of claim 4, said minimal origin of replication of papillomavirus being from BPV.

7. The method of claim 1 or the use of claim 4, further comprising a papillomavirus gene encoding E2.

8. The method of claims 1-3 or the use of claim 4, further comprising a papillomavirus gene encoding E1.

9. The method or use of claim 7 or 8 wherein said papillomavirus gene encoding E1 or E2 comprises a structural gene encoding E1 or E2 operatively associated with regulatory sequences for expression of the structural gene in a host cell.

10. The method or use of claim 9, said regulatory sequences comprising a promoter.

11. The method or use of claim 10, said promoter comprising a promoter that is non-native to said structural gene.

12. The method or use of claim 11, said promoter being functional in more than a single tissue type.

13. The method or use of claim 11, said promoter being functionally restricted to a single tissue type.

14. The method or use of claim 11, said promoter comprising one of the thymidine kinase promoter and the SR alpha promoter.

15. The method or use of claim 11, said promoter being a strong promoter.

16. The method of claims 1-3 or the use of claim 4, said vector further comprising a bacterial host cell origin of replication.

17. The method of claims 1-3 or the use of claim 4, said vector further comprising a gene encoding a selectable marker.

18. The method of claims 1-3 or use of claim 4 wherein said gene is operatively associated with regulatory sequences for expression of the gene in a host cell.

19. The method or use of claim 18, said regulatory sequences comprising a promoter.

20. The method or use of claim 19, said promoter comprising a promoter that is non-native to said structural gene.

21. The method or use of claim 20, said promoter being functional in more than a single cell type.

22. The method or use of claim 20, said promoter being functionally restricted to a single cell type.

23. The method or use of claim 7 wherein said gene encodes a mutant form of E2 which is replication competent but defective in transcriptional activation wherein said mutant form of E2

protein differs from the wild-type E2 in a nucleotide point mutation which translates into an amino acid substitution.

24. The method or use of claim 23 wherein said gene encoding said mutant form of E2 is operatively associated with a cell-type-restricted promoter.

25. The method or use of claim 23 wherein said gene encoding said mutant form of E2 is in trans to said minimal maintenance element and operatively associated with a cell-type-restricted promoter.

26. The method or use of claim 23, said amino acid substitution being R37A.

27. The method or use of claim 23, said amino acid being E74A.

28. The method or use of claim 23, said amino acid being D122A and D143A/R172C.

29. The method of claims 1-3 or the use of claim 4 wherein the minichromosomal maintenance element and the minimal origin of replication consists of a DNA sequence that is different from the natural papillomavirus sequence.

30. The method of claims 1-3 or the use of claim 4 wherein the minichromosomal maintenance element and the minimal origin of replication are separated by a distance of less than about 1.0 kb.

31. The method of claims 1-3 or the use of claim 4 wherein the minichromosomal maintenance element consists essentially of the region of BPV mapping to positions 7590 to 7673.

32. The method of claims 1-3 or the use of claim 4 wherein the minichromosomal maintenance element comprises (BPV E2 binding sites 6, 7 and 8) x, wherein x is 3 to 6.

33. The method of claims 1-3 or the use of claim 4 wherein the minichromosomal maintenance element comprises at least 2 of the 3 E2 binding sites 6, 7 and 8.

34. A vector for use in any one of claims 30-33.

35. A recombinant vector for stable maintenance of erogenous DNA in a eukaryotic host cell, the vector comprising

papilloma virus sequences consisting essentially of

(A) a minimal origin of replication of a papilloma virus,

(B) a minichromosomal maintenance element of a papilloma virus consisting essentially of at least two of the three E2 binding sites 6, 7, and 8,

wherein the region of the vector comprising the minimal origin of replication and minichromosomal maintenance element consists of a DNA sequence different from the natural papilloma virus sequence, and

wherein said vector, when present in a mammalian host cell which expresses E1 and E2, persists in said cell for at least about 50 cell generations in dividing cells or for at least about 8 weeks in non-dividing cells under nonselective conditions without an appreciable loss of copy number.

36. A recombinant vector for stable maintenance of erogenous DNA in a eukaryotic host cell, the vector comprising

papilloma virus sequences consisting essentially of

(A) a minimal origin of replication of a papilloma virus, and

(B) a minichromosomal maintenance element of a papilloma virus consisting essentially of multiple E2 binding sites,

wherein the distance between said minimal origin of replication and said minichromosomal maintenance element is less than about 1.0 kb,

wherein said vector, when present in a mammalian host cell which expresses E1 and E2, persists in said cell for at least about 50 cell generations in dividing cells or for at least about 8 weeks in non-dividing cells under nonselective conditions without an appreciable loss of copy number.

37. A recombinant vector for stable maintenance of erogenous DNA in a eukaryotic host cell, the vector comprising

papilloma virus sequences consisting essentially of

(A) a minimal origin of replication of a papilloma virus,

(B) a minichromosomal maintenance element of a papilloma virus consisting essentially of the region of BPV mapping to about positions 7590-7673

wherein said vector, when present in a mammalian host cell which expresses E1 and E2, persists in said cell for at least about 50 cell generations in dividing cells or for at least about 8 weeks in non-dividing cells under nonselective conditions without an appreciable loss of copy number.

38. A recombinant vector for stable maintenance of erogenous DNA in a eukaryotic host cell, the vector comprising

papilloma virus sequences consisting essentially of

(A) a minimal origin of replication of a papilloma virus, and

(B) a minichromosomal maintenance element of a papilloma virus consisting essentially of (BPV E2 binding sites 6, 7, and 8)x wherein x is 3 - 6,

wherein said vector, when present in a mammalian host cell which expresses E1 and E2, persists in said cell for at least about 50 cell generations in dividing cells or for at least about 8 weeks in non-dividing cells under nonselective conditions without an appreciable loss of copy number.

39. The recombinant vector of claims 35-38, said minichromosomal maintenance element of papillomavirus being from BPV.

40. The recombinant vector of claims 35-38, said minimal origin of replication of papillomavirus being from BPV.

41. The recombinant vector of claims 35-38, further comprising an expressible gene of interest.

42. The recombinant vector of claims 35-38, further comprising a papillomavirus gene encoding E1.

43. The recombinant vector of claims 35-38, further comprising an papillomavirus gene encoding E2.

44. The recombinant vector of claim 42 or 43 wherein said papillomavirus gene encoding E1 or E2 comprises a structural gene encoding E1 or E2 operatively associated with regulatory sequences for expression of the structural gene in a host cell.

45. The recombinant vector of claim 44, said regulatory sequences comprising a promoter.

46. The recombinant vector of claim 45, said promoter comprising a promoter that is non-native to said structural gene.

47. The recombinant vector of claim 46, said promoter being functional in more than a single cell type.

48. The recombinant vector of claim 46, said promoter being functionally restricted to a single cell type.

49. The recombinant vector of claim 46, said promoter comprising one of the thymidine kinase promoter and the SR alpha promoter.

50. The recombinant vector of claim 46, said promoter being a strong promoter.

51. Therecombinantvectorofclaims35-38,furthercomprisingabacterialhostcellorigin of replication.

52. The recombinant vector of claim 51, further comprising a gene encoding a selectable marker.

53. The recombinant vector of claim 41 wherein said gene comprises a structural gene operatively associated with regulatory sequences for expression of the structural gene in a host cell.

54. The recombinant vector of claim 53, said regulatory sequences comprising a promoter.

55. The recombinant vector of claim 54, said promoter comprising a promoter that is non-native to said structural gene.

56. The recombinant vector of claim 55, said promoter being functional in more than a single cell type.

57. The recombinant vector of claim 55, said promoter being functionally restricted to a single cell type.

58. The recombinant vector of claim 43 wherein said gene encodes a mutant form of E2 which is replication competent but defective in transcriptional activation, wherein the mutation is a point mutation.

59. The recombinant vector of claim 58 wherein said gene encoding said mutant form of E2 is operatively associated with a cell-type restricted promoter.

60. The recombinant vector of claim 58 wherein said gene encoding said mutant form of E2 is in trans to said minimal maintenance element and operatively associated with a cell-type-restricted promoter.

61. A host cell containing the vector of claims 35-60.

62. The host cell of claim 61, said cell being mammalian.

63. The host cell of claim 61, said mammalian cell being muscle or brain.

64. A method of obtaining stable long-term expression of a gene of interest in a cell, comprising providing the host cell of claim 61.

65. A method of obtaining stable expression of a gene of interest in a cell, comprising introducing into a host cell the recombinant vector of claims 35-38.

66. A method of treating a disease stemming from a genetic defect, comprising administering a therapeutically effective amount the vector of claims 35 -38 to a patient afflicted with said disease.

67. A method of manufacture of vector DNA, comprising culturing the host cell of claim 61.

68. A method of producing a protein of interest in a host cell, comprising culturing the host cell of claim 61 under conditions which permit expression of said gene of interest.

69. A method of producing a protein in a transgenic animal, comprising providing a transgenic animal containing the vector of claim 41 which produces the protein of interest.

70. A mutant form of a papillomavirus E2 protein wherein the replication function of said protein is competent and the transcriptional activation function of said protein is defective, wherein said mutant form of E2 protein differs from the wild-type E2 in a nucleotide point mutation which translates into an amino acid substitution.

71. The mutant form of a papillomavirus E2 protein of claim 70, said amino acid substitution being R37A.

72. The mutant form of a papillomavirus E2 protein of claim 70, said amino acid substitution being E74A.

73. The mutant form of a papillomavirus E2 protein of claim 70, said amino acid substitution being D 122A and D 143A/R172C.

74. A gene encoding the mutant form of the papillomavirus E2 protein of claim 70.

75. A host cell transformed with the gene of claim 74.

76. A kit for providing stable persistence of a vector in a host cell, the kit comprising the vector of claims 35-38 and packaging materials therefor.

77. A kit for providing a stably transformed host cell to a patient, the kit comprising the host cell of claim 61 and packaging materials therefor.

78. A kit for providing a mutant E2 protein to a host cell for stable persistence of a vector in the host cell, the kit comprising the mutant E2 protein of claim 70, and packaging materials therefor.

79. A kit for providing a mutant E2 protein to a host cell for stable persistence of a vector in the host cell, the kit comprising the gene of claim 74, and packaging materials therefor.

80. A mammalian disease model comprising a transgenic animal whose cells contain the vector of claim 41.

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(54) Title: EPISOMAL VECTOR AND USES THEREOF

(57) Abstract

The invention relates to a recombinant vector for stable persistence of erogenous DNA in a eukaryotic host cell, and the uses of the recombinant vector for long-term stable production of a gene product in the host cell, the vector including the minimal origin of replication of papillomavirus and the minichromosomal maintenance element of papillomavirus.

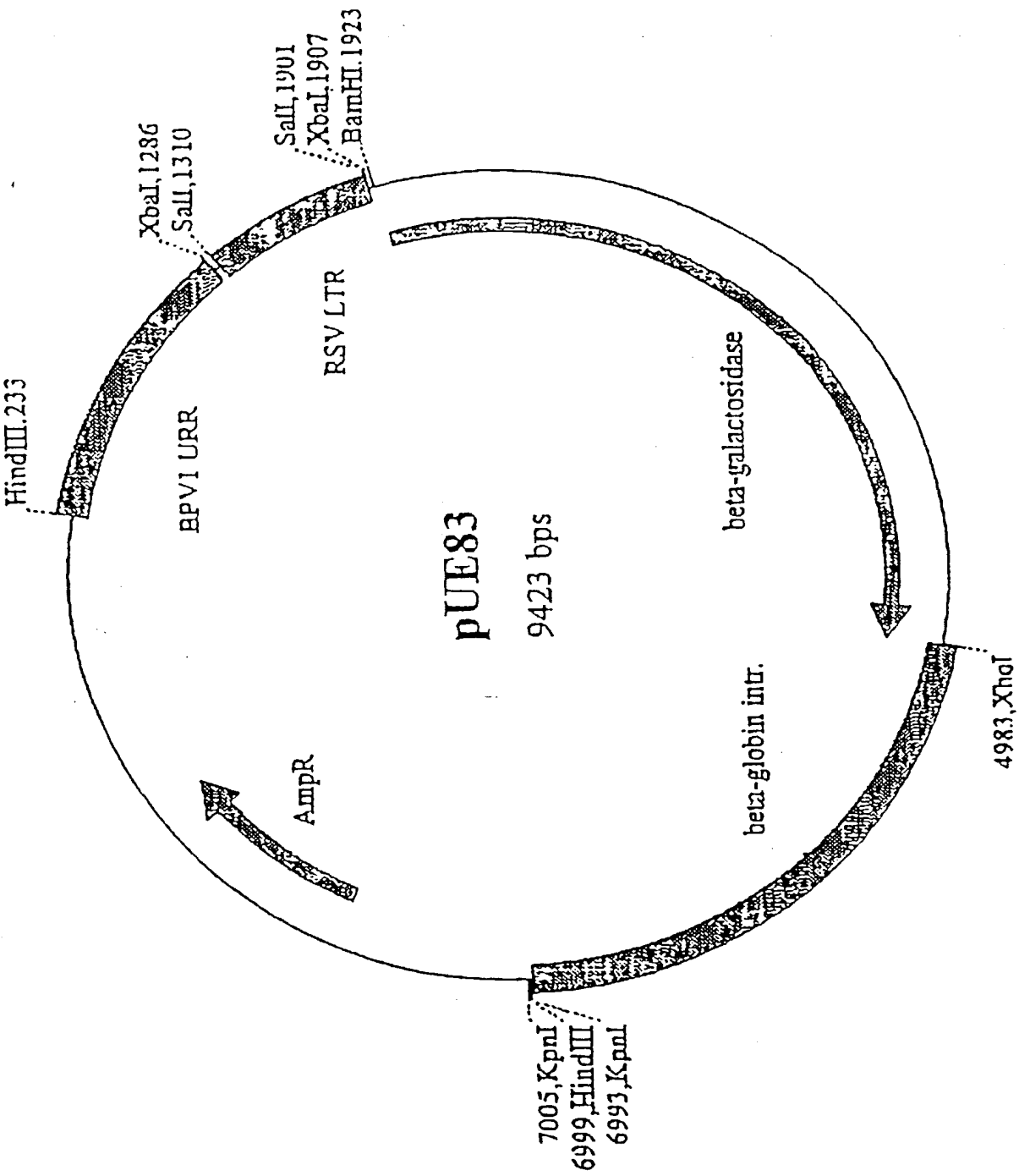
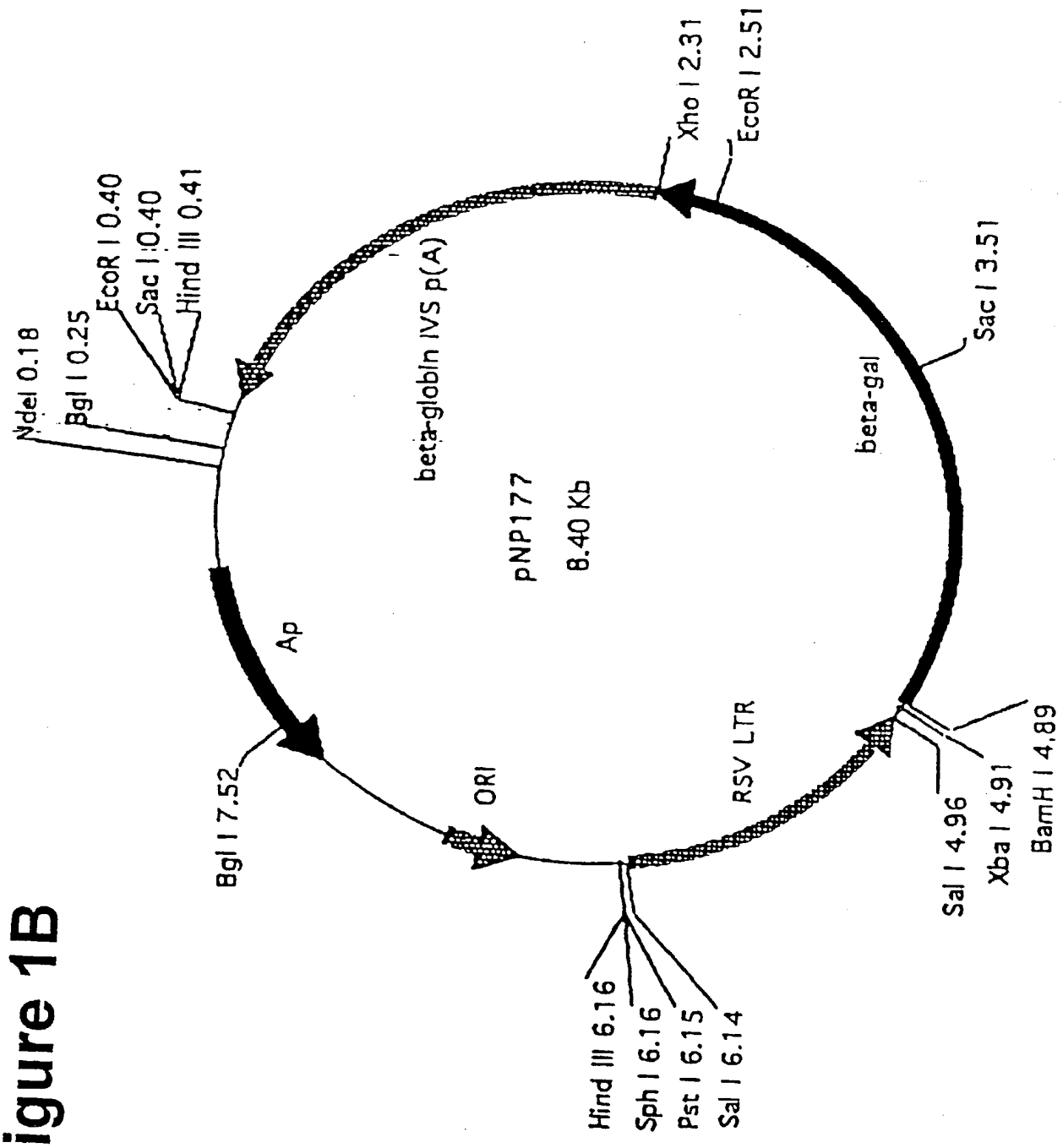

pUE83
9423 bps
RSV LTR
BPV1 URR
beta-galactosidase
AmpR
beta-globin intr.
HindIII,233
XbaI,1286
SalI,1310
SalI,1901
XbaI,1907
BamHI,1923
4983,XhoI
7005,KpnI
6999,HindIII
6993,KpnI

Figure 1A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Bgl I 0.25
EcoR I 0.40
Sac I 0.40
Hind III 0.41
beta-globin IVS p(A)
Xho I 2.31
EcoR I 2.51
Sac I 3.51
beta-gal
pNP177
8.40 Kb
Ap
Bgl I 7.52
ORI
RSV LTR
Hind III 6.16
Sph I 6.16
Pst I 6.15
Sal I 6.14
Sal I 4.96
Xba I 4.91
BamH I 4,89

Figure 1B

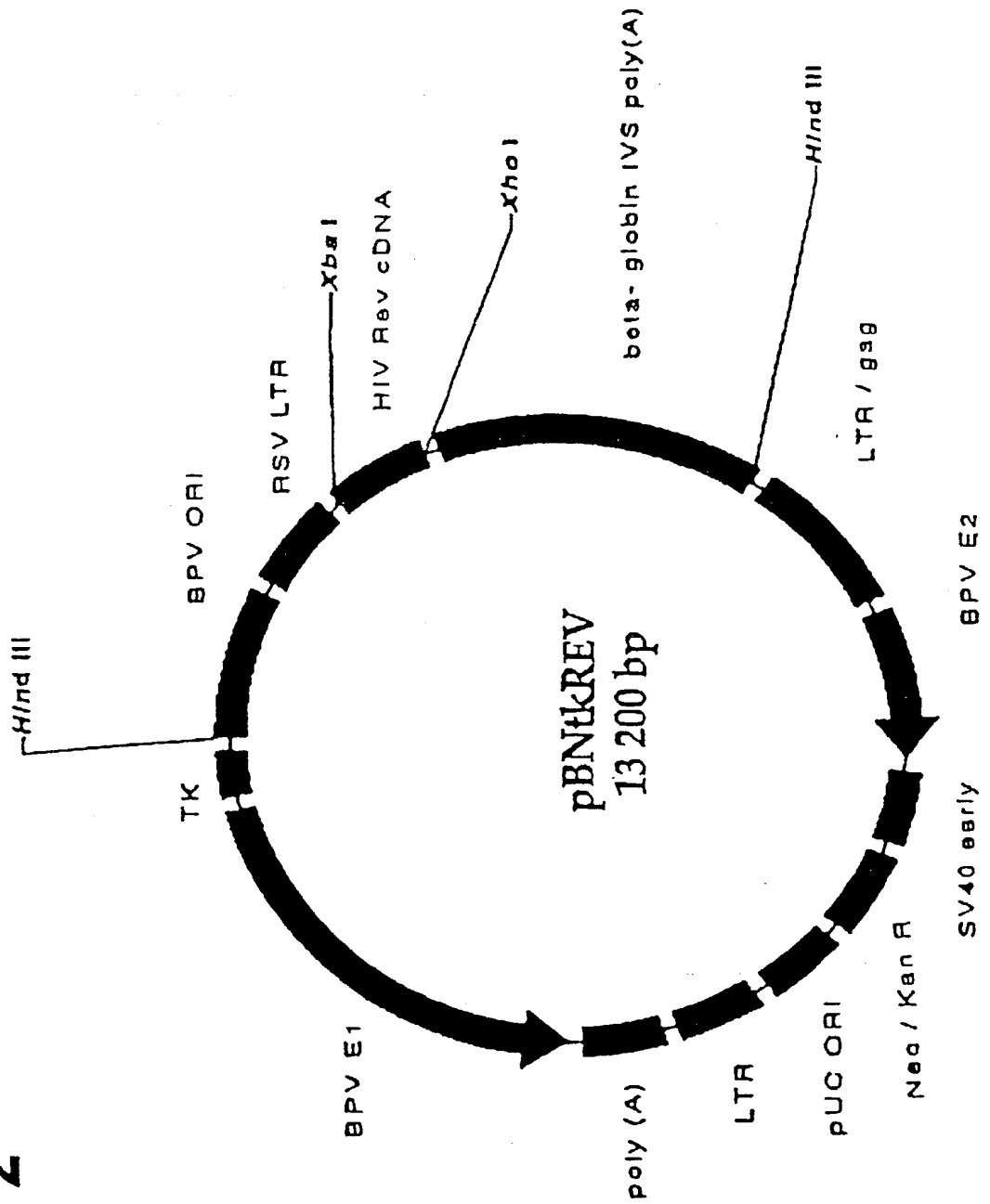
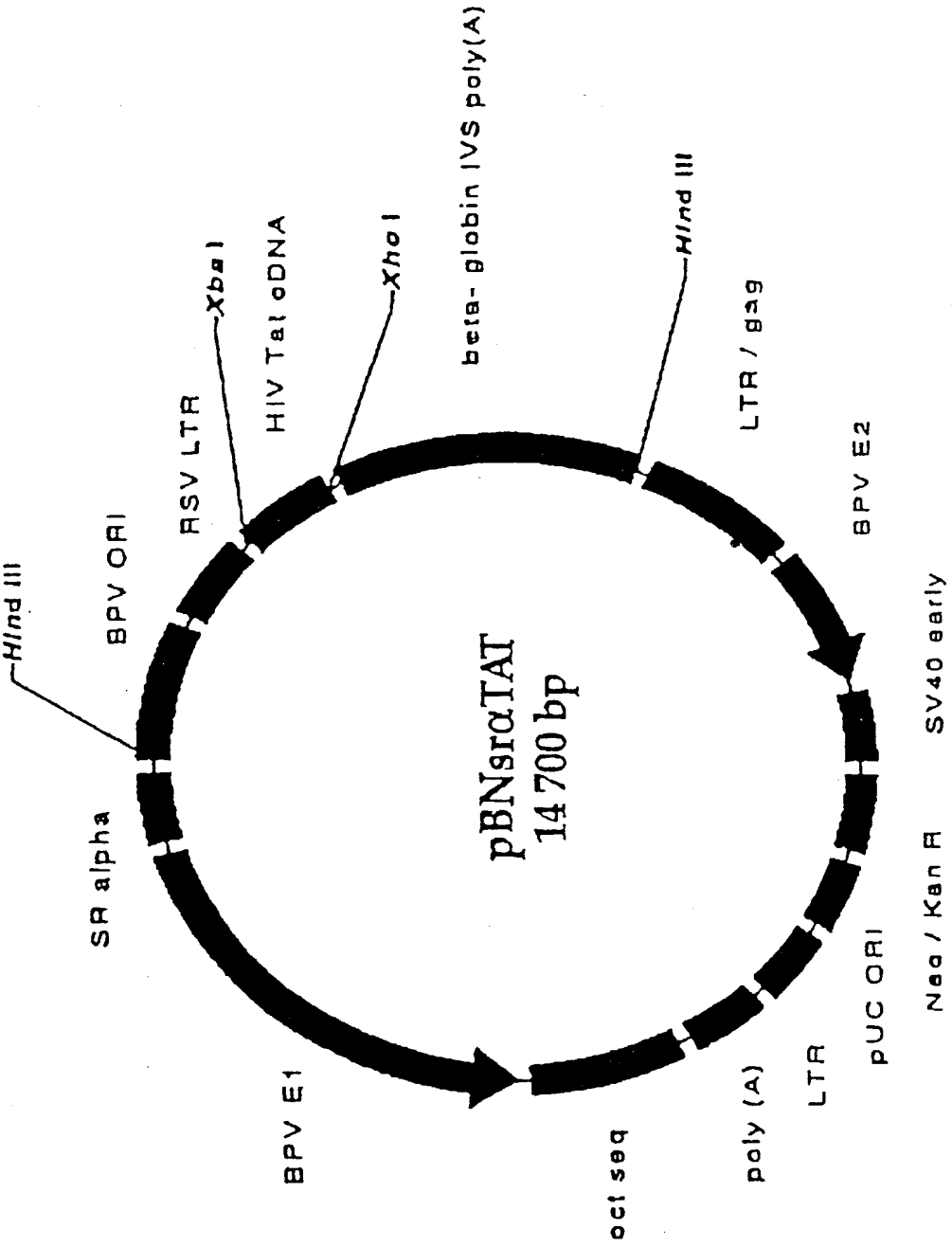

pBNtkREV
13 200 bp
Hind III
TK
BPV ORI
RSV LTR
Xba I
HIV Rev cDNA
Xho I
beta- globin IVS poly(A)
Hind III
LTR / gag
BPV E2
SV40 early
Neo / Kan R
pUC ORI
LTR
poly (A)
BPV E1

Figure 2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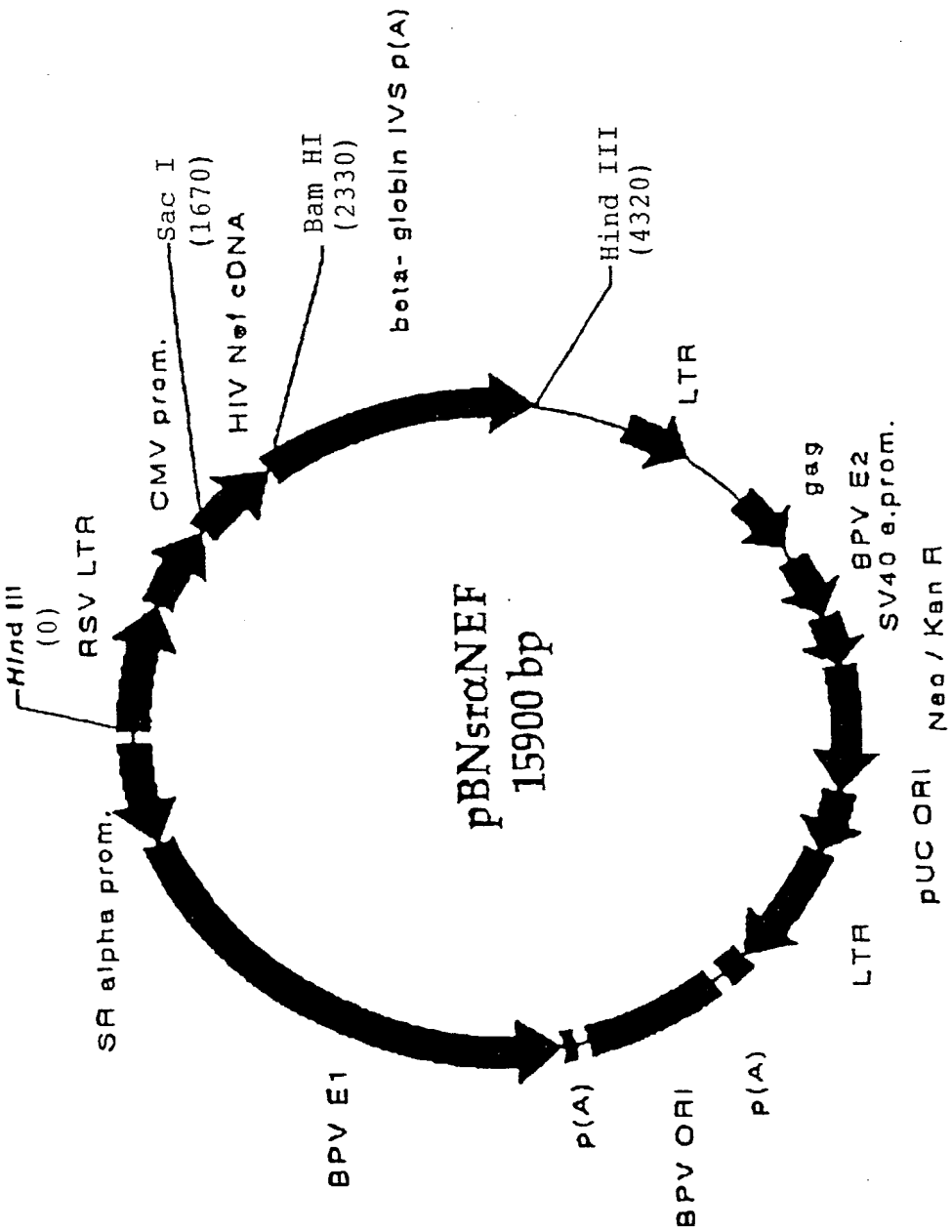
14 700 bp
Hind III
BPV ORI
RSV LTR
Xba I
HIV Tat cDNA
Xho I
beta- globin IVS poly(A)
Hind III
LTR / gag
BPV E2
SV40 early
Neo / Kan R
pUC ORI
LTR
poly (A)
oct seq
BPV E1
SR alpha

Figure 3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Hind III
(0)
RSV LTR
CMV prom.
Sac I
(1670)
HIV Nef cDNA
Bam HI
(2330)
beta- globin IVS p(A)
Hind III
(4320)
LTR
gag
BPV E2
SV40 e.prom.
Neo / Kan R
pUC ORI
LTR
p(A)
BPV ORI
p(A)
BPV E1
SR alpha prom.

Figure 4

Figure 5

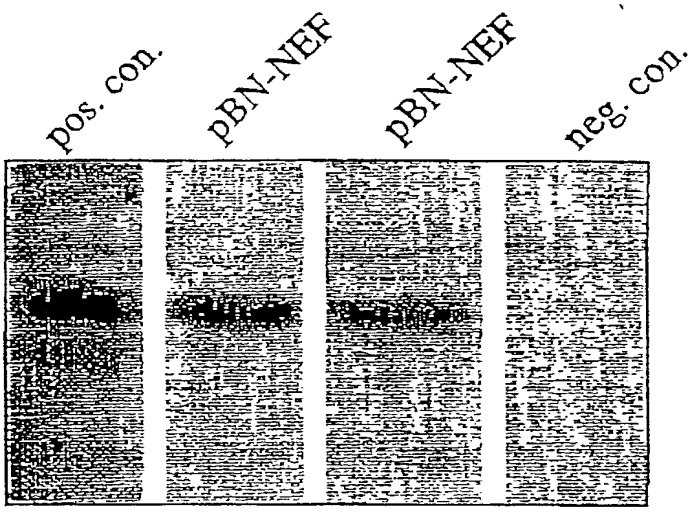


Figure 6

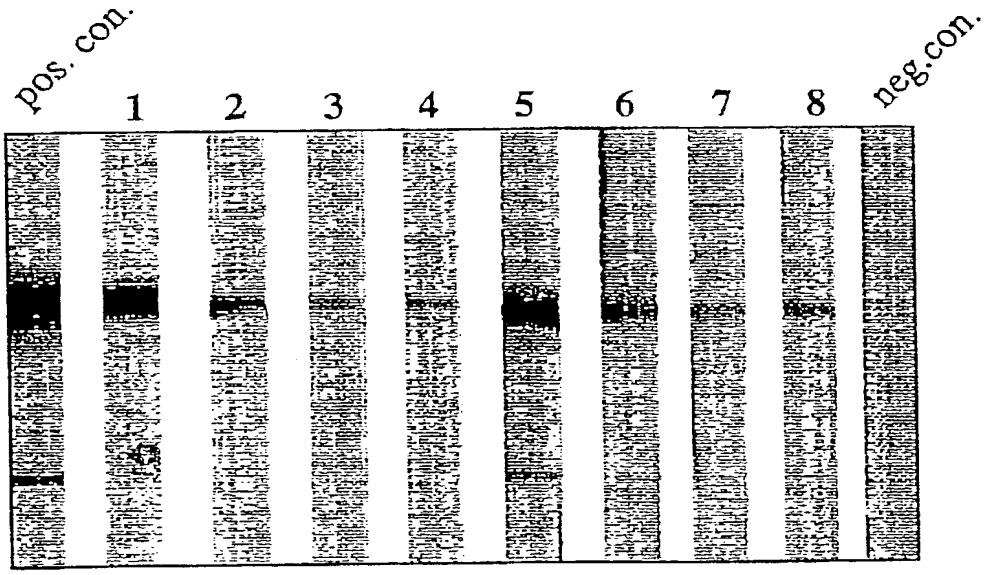


FIG. 7a

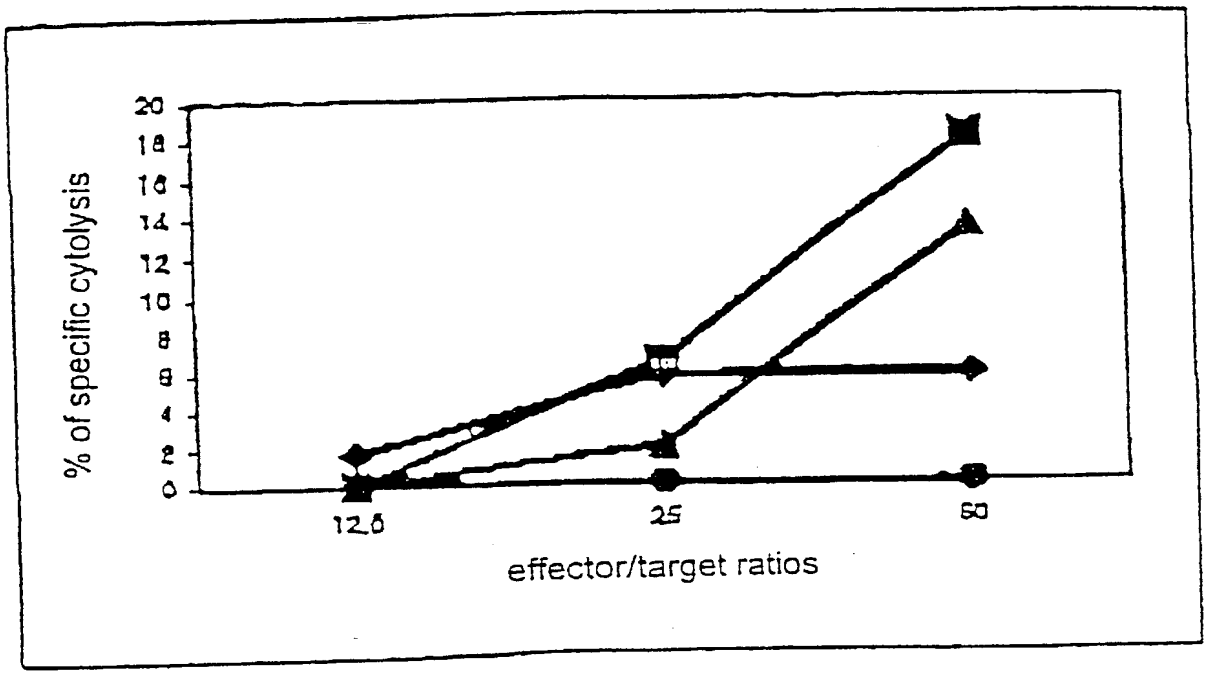

% of specific cytolysis
20
18
16
14
12
10
8
6
4
2
0
12,5
25
50
effector/target ratios

FIG. 7b

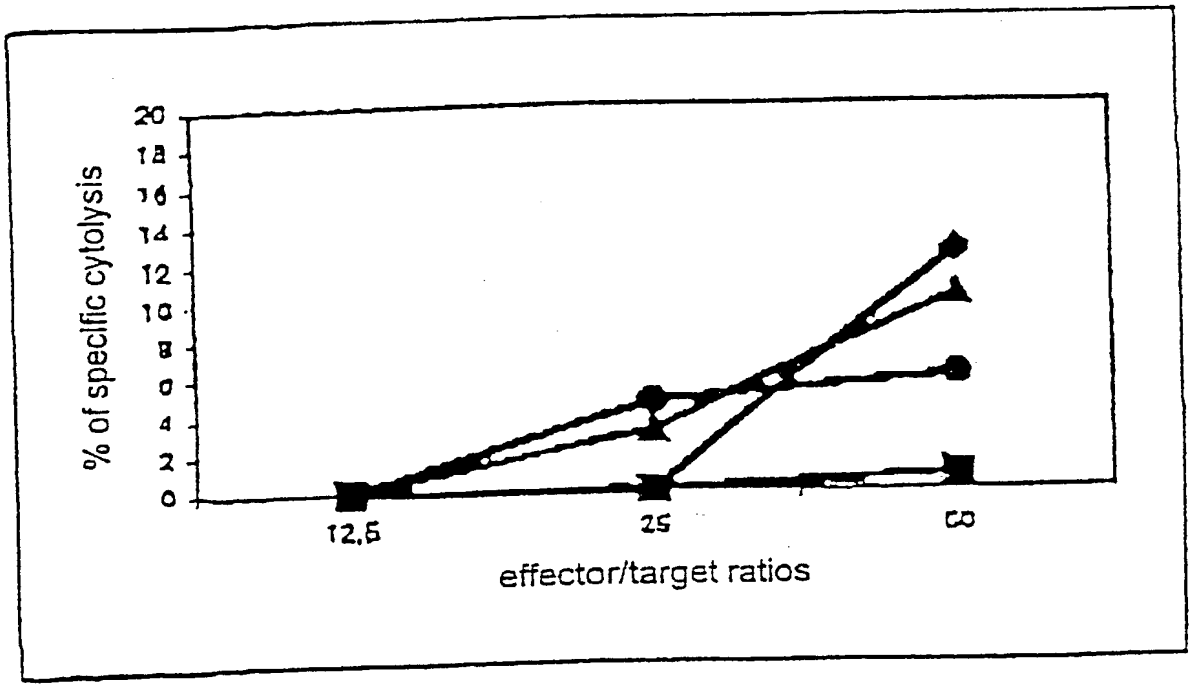
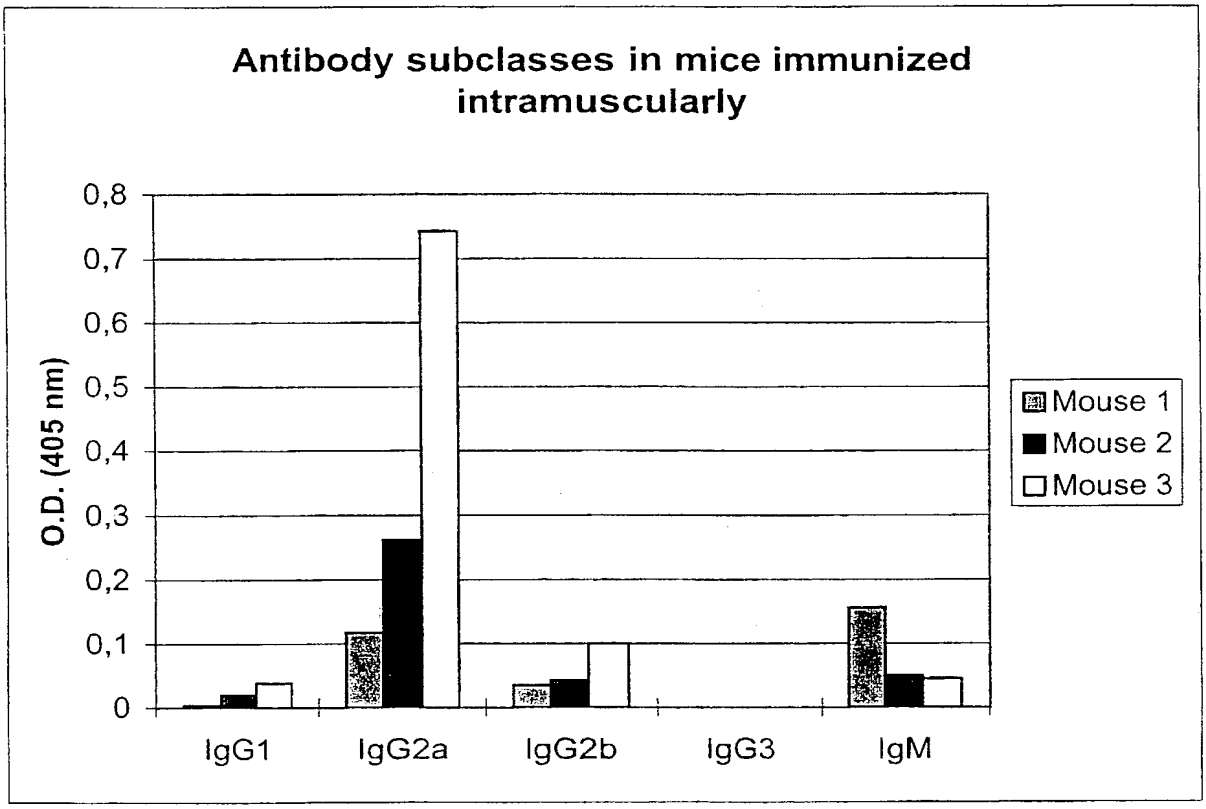

% of specific cytolysis
20
18
16
14
12
10
8
6
4
2
0
12,5
25
50
effector/target ratios

Figure 8


Antibody subclasses in mice immunized intramuscularly
O.D. (405 nm)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
IgG1
IgG2a
IgG2b
IgG3
IgM
Mouse 1
Mouse 2
Mouse 3

OMB No. 0651-0011 (12/31/86)

Applicant or Patentee: OY FINNISH IMMUNOTECHNOLOGY LTD. Attorney's Dkt. No. 227-135

Serial or Patent No.: 09/622,976

Filed or Issued:

For: Self-replicating vector for DNA immunization against HIV

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS [37 19(f) and 1.27(c)] - SMALL BUSINESS CONCERN

I hereby declare that I am

☐ the owner of the small business concern identified below:

☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN OY FINNISH IMMUNOTECHNOLOGY LTD.

ADDRESS OF CONCERN Lenkkeilijänkatu 8, FIN-33520 Tampere, Finland

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled:

Self-replicating vector for DNA immunization against HIV

by inventors Marja Tähtinen, Pärt Peterson, Kai Krohn and Päivi Annamari Ranki described in

☐ the specification filed herewith.

☒ application Serial No. 09/622,976 , filed

☐ patent No. , issued

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor who could not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 CFR 1.27)

Name

Address

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Name

Address

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

I acknowledge the duty to file, in this application of patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28(b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING PEKKA SILLANAUKEE

TITLE OF PERSON OTHER THAN OWNER CEO, MANAGING DIRECTOR

ADDRESS OF PERSON SIGNING Lenkkeilijänk. 8, FIN-33520 Tampere, FINLAND

SIGNATURE DATE 27. Sept. 2000

PEKKA SILLANAUKEE
CEO

Case No. _____

Nixon & Vanderhye P.C. (12/97)

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Self-replicating vector for DNA immunization against HIV

the specification of which (check applicable box(s)):

☐ is attached hereto
☐ was filed on _____ as U.S. Application Serial No. _____
☒ was filed as PCT International application No. PCT/FI99/00152 on 26 February 1999
and (if applicable to U.S. or PCT application) was amended on 21 February 2000

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Priority Foreign Application(s):

Application Number	Country	Day/Month/Year Filed
980463	Finland	27 February 1998

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

Application Number	Date/Month/Year Filed

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s): Application Serial No.	Day/Month/Year Filed	Status: patented pending, abandoned

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffry H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr. 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32956; J. Scott Davidson, 33489; Alan M. Kagen, 36178; William J. Griffin, 31260; Robert A. Molan, 29834; B. J. Sadoff, 36663; James D. Berquist, 34776; Updeep S. Gill, 37334.

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1-W

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Residence: (city) Tampere (state/country) Finland
Post Office Address: Kurunniitynkatu 2, FIN-33730 Tampere, Finland FIX
(Zip Code)

2W

Inventor's Signature: _____ Date: 27.9.2000
Inventor: Pärt (first) | MI | PETERSON (last) | Estonian (citizenship)
Residence: (city) Tampere (state/country) Finland
Post Office Address: Kaskitie 13 F, FIN-33540 Tampere, Finland FIX
(Zip Code)

3W

Inventor's Signature: _____ Date: 27.9.2000
Inventor: Kai (first) | MI | KROHN (last) | Finnish (citizenship)
Residence: (city) Salmentaka (state/country) Finland
Post Office Address: Salmentaantie 751, FIN-36450 Salmentaka, Finland FIX
(Zip Code)

FOR ADDITIONAL INVENTORS, check box ☒ and attach sheet with same information and signature and date for each.

264641

09622976 .100600

Case No. ___________

Nixon & Vanderhye P.C. (12/97)

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

4. Inventor's Signature: ______ Date: 27.9.2000
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Post Office Address: Sibeliuksenkatu 11 B 28, FIN-00250 Helsinki, Finland FIX
(Zip Code)

5. Inventor's Signature: ______ Date: ______
Inventor: (first) | MI | (last) | (citizenship)
Residence: (city) ______ (state/country) ______
Post Office Address: ______
(Zip Code) ______

6. Inventor's Signature: ______ Date: ______
Inventor: (first) | MI | (last) | (citizenship)
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Post Office Address: ______
(Zip Code) ______

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Inventor: (first) | MI | (last) | (citizenship)
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Residence: (city) ______ (state/country) ______
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